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U.S. Department of Health and Human Services

NTP Research Report on Biological Activity of Bisphenol A (BPA) Structural Analogues and Functional Alternatives

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The draft research report on the study of biological activity of bisphenol A (BPA) structural analogues and functional alternatives was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of these NTP studies was appropriate and ensured that this NTP Research Report presented the experimental results and conclusions fully and clearly.

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July 13, 2015: **Draft evaluation protocol reviewed:** sent to peer reviewers for comment/ review

August 14, 2015: Draft evaluation protocol finalized

August 18, 2015: **Two protocol revisions noted.** See "Searching Other Resources" and "Treatment of Special Content Types."

Abstract

Background: Recent studies report widespread usage or exposure to a variety of chemicals with structural or functional similarity to bisphenol A (BPA), referred to as BPA analogues or derivatives. These have been detected in foodstuffs, house dust, environmental samples, human urine or blood, and thermal paper. Compared to BPA relatively little is known about potential toxicity of these compounds.

Objective: To identify and summarize human, animal, and mechanistic toxicity data for 24 BPA analogues of emerging interest to research and regulatory communities.

Methods: The objective was addressed by two efforts: 1) a systematic review of the available research; and 2) analysis of data available from the high throughput screening programs Tox21/ToxCast. We used systematic review methods to identify relevant studies from the published literature. Over 5,100 literature studies were screened for relevance and 166 were considered relevant. Analyses of the high throughput screening data focused on assessing structural and biological similarity among the BPA analogues and between BPA or estradiol (E_2).

Results: Reports on 16 of the 24 analogues were found in the published literature. There were no studies of human health effects, animal toxicity or mechanistic studies for 8 of the 24 compounds. The only human health effect that was reported was dermal sensitization to 4,4-BPF, 2,2-BPF or BPS. The majority of the available research was conducted *in vitro.* Analysis of the Tox21/ToxCast data showed that in general, BPA analogues and derivatives are more structurally and biologically similar to BPA, and to each other, than to E_2. Taken together, the published literature and the data available in Tox21/ToxCast demonstrate that many of the BPA analogues that are potential replacements for BPA have biological activity within the range of activity observed for BPA.

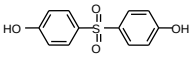
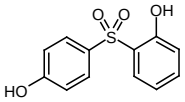
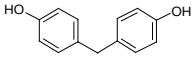
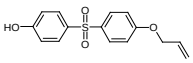
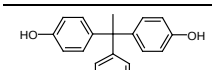
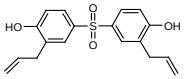
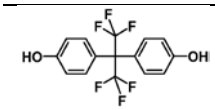
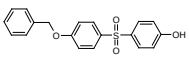
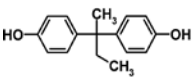
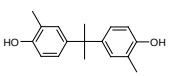
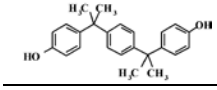
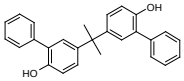
Conclusion: The results of these analyses suggest that many of these chemicals may have endocrine activity *in vivo*. Given that these chemicals have potential widespread use, they should be pursued in further testing and reconsidered as appropriate replacements for BPA in consumer products.

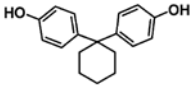
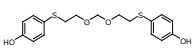
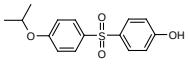
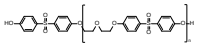
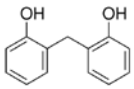
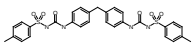
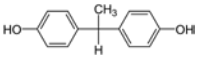
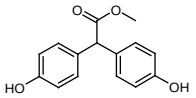
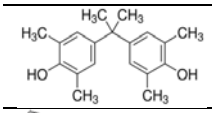
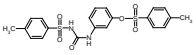
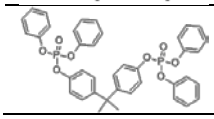
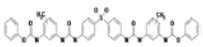
Introduction

Background

Bisphenol A (BPA) is a high production volume chemical used in the manufacture of polycarbonate plastics, epoxy resins, as a dye developer in thermal paper, and as a polymerization inhibitor in the formation of some polyvinyl chloride plastics[38; 40; 103]. Polycarbonates are in consumer products such as plastic dinnerware, microwave ovenware, eyeglass lenses, toys, pacifiers, impact-resistant safety equipment, compact discs and automobile parts. Epoxy resins are used in protective linings of canned food and beverage containers, drinking water storage tanks, wine vat linings, some paints, floorings, and some dental composites[38; 40; 103]. The types of thermal paper products where BPA might be used as a developer include cash register receipts and certain medical technical paper[38; 108]. Consequently, human exposure is widespread. BPA has been detected in the urine of 92% of participants surveyed in the United States National Health and Nutrition Examination Survey (NHANES) in 2003 to 2004[156]. BPA has been reported to cause a wide range of adverse health outcomes in experimental animal studies; similar findings in humans have also been linked to BPA exposure in observational epidemiology studies[40; 103; 115; 143].

Table 1. BPA Analogues Included in Systematic Review

Structure	Abbreviation (CASRN)	Detection	Structure	Abbreviation (CASRN)	Detection
	BPS (80-09-1)	blood[88], food[92], dust[89], sediment[91], receipts[6; 7; 90; 140], urine[162; 163]		2,4-BPS (5397-34-2)	*receipts*[140]
	4,4-BPF (620-92-8)	food[14; 52; 92], dust[89], sediment[91], *receipts*[140], urine[162; 163], PCP[15], municipal sewage sludge[127]		BPS-MAE (97042-18-7)	*receipts*[140]
	BPAP (1571-75-1)	food[92], dust[89], sediment[91], *receipts*[140]		TGSA (41481-66-7)	*receipts*[140]
	BPAF (1478-61-1)	food[92], dust[89], sediment[91], municipal sewage sludge[127]		BPS-MPE (63134-33-8)	*receipts*[140]
	BPB (77-40-7)	food[23-25; 51; 52; 92], dust[89], sediment[91], blood[19; 20], urine[22]		BPC (79-97-0)	*receipts*[140]
	BPP (2167-51-3)	food[92], dust[89]		BPPH (24038-68-4)	*receipts*[140]

Structure	Abbreviation (CASRN)	Detection	Structure	Abbreviation (CASRN)	Detection
	BPZ (843-55-0)	food[14; 92], sediment[91], PCP[15]		DD-70 (93589-69-6)	*receipts*[140]
	D-8 (95235-30-6)	blood[134], receipts[134]		D-90 (191680-83-8)	*receipts*[140]
	2,2-BPF (2467-02-9)	resins[11]		BTUM (151882-81-4)	*receipts*[140]
	BPE (2081-08-5)	municipal sewage sludge[127]		MBHA (5129-00-0)	*receipts*[140]
	TMBPA (5613-46-7)	polycarbonate resin[79]		Pergafast 201 (232938-43-1)	*receipts*[140]
	BDP (5945-33-5)	*flame retardant*		UU (321860-75-7)	*receipts*[140]

Italics indicate that this chemical has been suggested for use as described, but its use or detection has not been confirmed. Full chemical names can be found in **Supplemental Table 1**. **Abbreviations**: PCP (personal care products).

Recent studies report widespread exposure to a variety of chemicals with structural or functional similarity to BPA, often referred to as BPA analogues or derivatives (and henceforth referred to as BPA analogues) (Table 1). BPA analogues have been detected in foodstuffs[14; 92], house dust[89], river and lake sediment[91], personal care products[93], and thermal paper[7; 90]. Importantly, BPA analogues have also been detected in human biological specimens[19; 20; 22; 88; 162; 163]. Several chlorinated and brominated derivatives of BPA are used as flame retardants[38; 40; 103; 144]. Other chemicals (e.g. MBHA) have also been identified as theoretical alternatives to BPA in thermal paper, although the extent to which they are actually being used is not known[140].

In contrast to BPA, most BPA analogues are poorly understood with respect to potential toxicity[104; 140]. Use of these compounds may increase as companies move towards using alternatives to BPA in consumer products[41; 42; 44]. The health effects of two BPA analogues, bisphenol S and bisphenol F (BPS and BPF) have been recently reviewed using systematic review methodology[116], and the United States Environmental Protection Agency's (US EPA) Design for the Environment program completed an alternative assessment in January 2014 in which the potential human health and environmental impacts of chemical alternatives to BPA in thermal paper were summarized[140]. However, the literature for some analogues is growing rapidly.

Objectives

The objective of this review is to answer the question: "What is the biological activity of BPA analogues of emerging public health concern?" Our specific aims were to:

- identify all of the publicly available human, animal, and *in vitro* literature concerning health outcomes or biological responses of BPA analogues with the highest potential for human exposure (Table 1);
- extract data from the relevant studies;
- assess the risk of bias of individual animal and human studies;
- synthesize and summarize the existing evidence based on associated health outcome or biological response;
- evaluate the structural and biological similarity of the analogues to each other, to BPA, and to the potent estrogen estradiol (E_2) within the National Toxicology Program's (NTP) Tox21 and US EPA's ToxCast high throughput screening (HTS) platforms, and
- identify data gaps and research needs that could aid in assessment or development of BPA alternatives.

Methods

Systematic Review Methods

Methods for the systematic review are briefly summarized below and are available in more detail in the evaluation protocol[105]. Systematic review processes were conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement criteria[97] and following the OHAT framework for conducting a systematic review[119].

Formulate the Study Question

A literature search strategy was initially developed for 27 BPA structural and/or functional analogues and refined during problem formulation (described in the evaluation protocol) to focus on the 24 BPA analogues listed in Table 1 (with additional details provided in Supplemental Table 1). In brief, analogues were prioritized for inclusion in the systematic review based on (1) detection in the environment (e.g., dust, water, sewage), foodstuff, or human biological samples; (2) identification by the US EPA Design for the Environment (DfE) program as being a potential alternative to BPA in thermal paper (henceforth referred to as the "US EPA DfE report")[140]; (3) use as a halogenated flame retardant; and (4) considered of emerging interest, i.e. relatively data-poor and not the focus of many previous or on-going hazard or risk evaluations.

To address our overall objective we developed a PECO (Populations, Exposures, Comparators, Outcomes) statement (Table 2) to aid in developing an answerable question, the search terms, and the inclusion/exclusion criteria for our systematic review[1; 60]. The overall objective and PECO statement were based on a series of problem formulation steps that included assembling an NIEHS/NTP evaluation design team with expertise in BPA and BPA alternatives, toxicology, epidemiology, systematic review, information science, and analysis of high throughput screening data; and consultation with scientists at state governments and other Federal agencies. More details about problem formulation activities can be found in the evaluation protocol[105].

Table 2. PECO (Populations, Exposures, Comparators Outcomes) Statement

PECO Element	Evidence
Populations	Human, animal (whole organism), or *ex vivo/in vitro* models utilizing organs, tissues, cell lines, or cellular components (*e.g.* cell-free receptor binding assays).
Exposures	Exposure to at least one of the 24 BPA analogues listed in Table 1.
Comparators	Humans, animals, organs, tissues, cell lines, or cellular components exposed to a lower level of a BPA analogue than the more highly exposed subjects or treatment groups, or vehicle-only treatment.
Outcomes	Any health outcome or type of biological response.

Search For and Select Studies for Inclusion

Literature Search Strategy

Prior to the literature search, the SciFinder database was searched using each chemical's chemical abstracts service registration number (CASRN) to retrieve synonym names as well as old or additional CASRNs. Six databases were initially searched on March 28, 2014: Embase, PubMed, SciFinder, Scopus, Toxline and Web of Science. The search strategy was customized for each database because of differences in syntax. In addition, a broader search of the literature using the phrase "bisphenol A (analogue or analog)" was done in order to retrieve studies that had not specified analogues in the title or abstract. The search was not limited by language or publication date. A literature search update was performed on March 23, 2015. The search update did not include searches for PHBB, TBBPA, or TCBPA because these compounds were not prioritized after problem formulation as they have been the focus of prior literature-based evaluations. The specific language used for each database and numbers of studies retrieved are available in the evaluation protocol.

Searching Other Resources

Additional relevant publications were searched for by reviewing studies from the database search that did not contain original data (e.g., reviews), the US EPA DfE report[140], a NTP background document on BPA alternatives[104], and the European Chemicals Agency (ECHA) database that contains registration dossiers submitted for REACH chemicals (https://echa.europa.eu/information-on-chemicals/registered-substances). Though the ECHA database was searched, relevant data could not always be analyzed to the same degree as other included studies because the information is not peer reviewed and the database often only contains summaries of study findings. For example, the study's No Observed Effect Level (NOEL) may be listed, but the study summary does not present underlying data such as mean or standard deviation. When available, relevant findings from studies in ECHA are presented separately from those available in the published literature. Additionally, the numbers of studies available in ECHA for each chemical and study type are listed in the results tables in order to indicate the availability of the data.

Study Selection Criteria

Studies were screened for inclusion using a structured form in DistillerSR (Evidence Partners; http://www.evidencepartners.com). In order to be eligible for inclusion, studies needed to comply with the criteria specified by the PECO statement (Table 2). Studies that did not meet the PECO criteria were excluded. In addition, the following exclusion criteria were applied: studies

that did not contain original data, such as reviews, editorials, or commentaries, or that were conference abstracts.

Two members of the evaluation design team (K.E.P. and K.A.T. or P.R.) independently conducted title and abstract screening of the search results to determine whether a reference met the inclusion criteria; studies not excluded based on the title and abstract were screened through a full-text review. Full-text copies of potentially relevant studies were reviewed by one author (K.P.) and exclusions confirmed by a second author (K.A.T. or P.R.). Discrepant screening results were resolved by discussion. Native-language speakers at NIH facilitated the assessment of eligibility status of non-English studies.

Although beyond the scope of the current review, we also tracked studies reporting information on human exposure (detection in human blood, urine, or tissues) (Supplemental Table 2); wildlife (Supplemental Table 3); absorption, distribution, metabolism, and excretion (ADME) (Supplemental Table 4); and studies assessing the health effects of PHBB, TCBA, and TBBPA, which were not included past problem formulation as they have been the focus of prior literature-based evaluations (Supplemental Table 5, Supplemental Table 6, Supplemental Table 7, respectively).

Extract Data from Studies

Data were extracted from studies that met the inclusion criteria using DRAGON software[64; 65]. For each study, data extraction was done by one of the contract staff and reviewed for completeness and accuracy by a senior contractor (J.W.) and by K.E.P. The following data were collected for each study: authors, journal, reference information, year of publication, chemical, purity, dose or concentration tested, species/strain, cell line/tissue type, assay, endpoint, and AC_{50} (when reported). Dose and concentration level specific effects were summarized based on statistical significance as reported in the research studies. For *in vivo* studies, measures of effect at each dose or concentration level were extracted (e.g., mean, median, and measures of precision or variance). Findings from *in vivo* studies for continuous endpoints were converted to percent of control response using numbers presented in tabular form or by use of the WebPlot Digitizer[118] from graphical depictions of data. For *in vitro* studies, the effects were summarized as increase, decrease, or no change from a vehicle control at each dose tested. One limitation is that in the *in vitro* studies authors often express results as a percentage of the positive or negative control response which may miss subtle induction or inhibition effects only apparent when looking at raw data. Disagreement was resolved by discussion between reviewers. Data extraction results are stored and visualized using Health Assessment Workspace Collaborative (HAWC)[122] and are available for download in Excel format (https://hawcproject.org/assessment/46/).

Assess Internal Validity ("Risk of Bias") of Individual Human and Animal Studies

Internal validity, often referred to as "risk of bias" in systematic review, was assessed for individual human and animal studies using the OHAT risk of bias tool[106; 119]. In brief, risk of bias questions addressed randomization, allocation concealment, similarity of conditions across groups, blinding during the study, characterization of the treatment, adequacy of the outcome assessment, concerns for missing data, and other potential threats to internal validity such as

failure to control for litter effects in developmental studies. Each risk of bias questions was answered on a 4-point scale: "definitely low risk of bias," "probably low risk of bias," "probably high risk of bias," and "definitely high risk of bias." Risk of bias was independently assessed by one senior contract staff (R.B.), reviewed by another (P.R), with a final review conducted by K.E.P. Any discrepancies were resolved by consensus, arbitration by an additional member of the review team, or consultation with technical advisors as needed. Risk of bias was not assessed for *in vitro* studies because a tool analogous to that used for human and animal studies has not yet been finalized for the NTP method.

Structural and Biological Similarity Analysis

Structural Similarity Analysis

Chemical structures (SMILES strings) were retrieved from the US EPA annotation of the Tox21 10K library[141] (Supplemental Table 1). Structures were not available for two BPA analogues that are polymers (D90 and UU). For chemicals that were not contained in the Tox21 library a SMILES string was identified in the EPA Chemical Dashboard (https://comptox.epa.gov/dashboard) by searching with CASRN. Smiles strings were loaded into the Leadscope Model Applier software (Version 3.1; Columbus, OH) and the presence or absence of a total of 8,026 structural features representing medicinal chemistry building blocks were annotated to the chemicals. Tanimoto coefficients (chemical structure similarity metric) for the structural features were then calculated for all pairs of chemicals. Pairs of chemicals with Tanimoto coefficients close to 1 are quite similar and those close to 0 have limited similarity.

HTS Data

Analyses of HTS data from Tox21[136] and ToxCast[71] focused on evaluating structural and biological similarity among the BPA analogues and relative to BPA or E_2. Fifteen BPA analogues (TGSA, BPC, 4,4-BPF, TBBPA, PHBB, BPS, TCBPA, 2,2-BPF, BPE, TMBPA, BPAF, BPZ, BPB, BPPH, BPS-MPE) were included in the Tox21 library, 4 (TGSA, TBBPA, BPAF, BPB) were included in the ToxCast Phase I/II libraries, and 6 were included in the ToxCast E1K library (PHBB, BPS, TCBPA, 2,2-BPF, BPB, BPAF) (Supplemental Table 1), which is an additional set of 800 compounds that are part of the Endocrine Disruption Screening Program (EDSP21) and that have only been tested in the endocrine-related subset of ToxCast assays[139]. Although not included in the systematic review, the "data rich" chemicals TBBPA, TCBPA, and PHBB were included in the HTS similarity profiling.

Biological Similarity Analysis Based on HTS Data

The biological similarity was assessed either in the scope of BPA analogues themselves (analysis #1), in the scope of pharmaceutical estrogens (analysis #2) or in the scope of the whole Tox21 library (analysis #3). In the first analysis, the activity values (AC_{50}; concentration eliciting a half-maximal response) of chemicals in Tox21 assays were retrieved from https://ntp.niehs.nih.gov/sandbox/tox21-activity-browser/[63]. Activities that were potentially confounded by interferences such as compound auto-fluorescence and compound cytotoxicity were flagged as inconclusive[63]. The similarity of chemicals was viewed as a dendrogram based on the results of hierarchical clustering with average linkage using AC_{50} values (-log10(M), 1M is set for inactives or inconclusives). At the time of analysis, 24 assays targeting nuclear receptor

signaling pathways and stress response pathways were used (Supplemental Table 8). Most Tox21 assays detect the target of transcriptional factor activity using reporter gene transcription technology (https://ncats.nih.gov/tox21/projects/nrassays). BPA and E_2 were included for comparison. The ToxPI tool[113] was used to compare the potency (AC_{50}) between BPA analogues, BPA, and E_2, relative to the most potent chemicals in the library (Supplemental Table 8).

In the second analysis, the activity values (wAUC, weighted area under the curve)[63] from 55 assays (cell viability assays included) including 441 outputs were used. The activity of the BPA analogues was first compared to that of a set of Tox21 chemicals similar to E_2, then more broadly to the entire Tox21 chemical library. The E_2 set was defined as all of the Tox21 chemicals with Tanimoto similarity to beta-estradiol of greater than 0.5 (see Supplemental Materials).

In a third analysis, similarity to BPA or E_2 was determined for the BPA analogues in Tox21 in addition to all other chemicals run as part of Tox21. Log transformed +1 wAUC values from a total of 441 assay metrics across 55 assays were used to determine the Pearson correlation coefficient between E_2 or BPA and chemicals in the Tox21 library. Assays for which there were no data for one or both of the chemicals were omitted from the calculation. A bootstrap resampling method was used to calculate the 95% confidence intervals. For each pair of chemicals, a bootstrap sample was generated by resampling with replacement from the pairs of wAUC values for those chemicals, and the correlation coefficient for these resampled values was generated. This procedure was performed 1,000 times. If the resampled data could not be used to compute a correlation coefficient (e.g. if all data points had wAUC values of 0 (i.e., inactive) for both assays), the value of the correlation coefficient was treated as 0. The 95% confidence limit was calculated as the range from the 2.5^{th} to 97.5^{th} percentiles of the bootstrapped values. P values for the correlation coefficients were also calculated. The p value of the observed correlation was calculated as the fraction of the bootstrapped correlation with absolute values greater than the observed correlation value, under the null hypothesis of no correlation. Chemicals with less than high quality (“A” rating for purity, defined as >90% and molecular weight identity confirmed) were removed from the analysis to avoid spurious results. For visualization purposes correlation values represent an average by CASRN when the chemical was present more than once in the library (i.e., the chemical had multiple Tox21 IDs).

Integrated Chemical and Biological Similarity

Chemical structural similarity data from above was merged with biological similarity data from the biological similarity analysis #3 to yield a plot illustrating a trend of increasing biological similarity with chemical similarity for the BPA analogues in the context of the entire Tox21 library.

Results

Literature Search Results

The literature searches yielded 5,124 unique studies and an additional 8 studies were identified while reviewing included studies. Of these, 4,783 were excluded during title and abstract screening. Three hundred forty nine studies were reviewed at the full text level, and 166 were

identified as relevant to the PECO statement (Figure 1). Nine of the 166 studies (5%) were non-English[4; 86; 100; 145; 149; 157; 159-161].

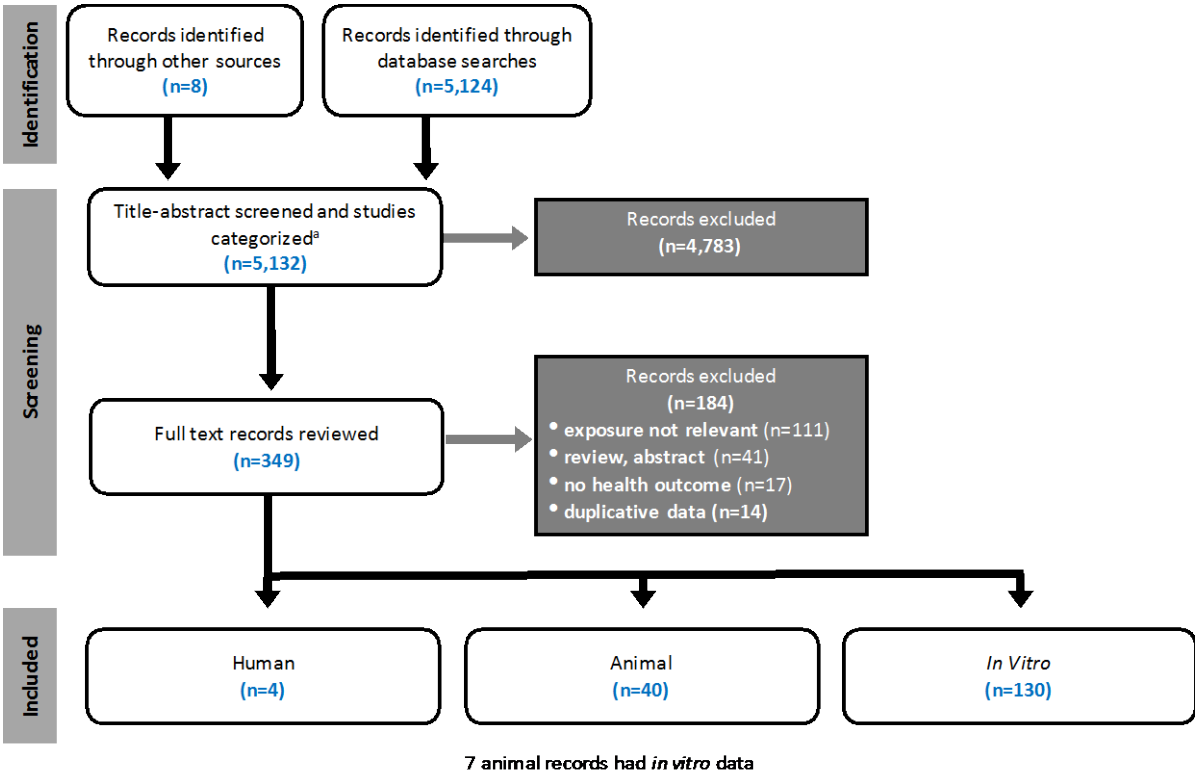


Figure 1. Study Flow Diagram

Study flow diagram. Describes the number of studies processed at each step of the evaluation. [a]Records were broadly characterized by evidence stream (human, animal, *in vitro*), type of health outcome, and chemical. No results are extracted or summarized.

There was very little to no toxicity data available for many of the BPA analogues. Sixteen of the 24 BPA analogues had been reported on in at least 1 publication, but only ten had been evaluated in more than three studies. Most studies reported on *in vitro* data (n=130), whereas fewer (n=40) reported on *in vivo* data and only 4 studies reported on human epidemiological data (Figure 1, Table 3). There were no studies recovered from the literature search for eight of the 24 BPA analogues (BPS-MAE, BPS-MPE, BTUM, D-90, DD-70, MBHA, TGSA, UU). These eight BPA analogues were listed in the 2014 US EPA DfE Report where their potential hazards were summarized based largely on values from estimation software, professional judgment, analogy to experimental data for a structurally similar compound, or, where available, submitted confidential studies[140] (Supplemental Table 11). One of these chemicals (BPS-MAE) as well as BDP, BPS, and Pergafast 201 were included in the ECHA database (See Supplemental Materials).

Survey of Evidence

Human Evidence

Four human studies reported information on exposure to BPA analogues and contact dermatitis[10; 55; 67; 125]. No other health outcomes were assessed in the human studies. In the four human studies patients or workers with suspected allergic contact dermatitis were patch tested with one or more of the BPA analogues or additional chemicals that they may have come in contact with including plastics, epoxy resins, glues, and hardeners. Dermal patch testing with BPF (both the 2,2- and 4,4-isomers) caused some reactions in patients or workers[10; 55; 125]. In contrast, dermal patch testing with BPS did not elicit an effect[67].

Table 3. Inventory of Published Literature by Chemical and Evidence Stream

Chemical	Human	Animal	In Vitro
4,4-BPF	3	15	61
BPS	1	9	52
BPAF	0	10	41
BPB	0	9	35
BPC	0	5	22
BPE	0	3	23
BPZ	0	3	15
TMBPA	0	1	14
BPAP	0	2	9
BPP	0	0	6
2,2-BPF	0	2	1
BDP	0	1	2
2,4-BPS	0	1	1
BPPH	0	0	2
D-8	0	0	2
Pergafast 201	0	0	1

Animal Evidence

Forty experimental animal studies reporting on 13 BPA analogues were identified from the published literature and are summarized in Table 4 following the definitions provided in[140] . The most commonly studied BPA analogues were 4,4-BPF (n=15 studies), BPAF (n=10 studies), BPB (n=9 studies) and BPS (n=9 studies). Eleven studies assessed uterotrophic responses (4,4-BPF, BPS, BPZ, BPB, 2,2-BPF, BPAF, BPC, BPAP), five studies assessed subchronic exposure (BPAF, 4,4-BPF, BPZ, BPS), eleven studies assessed sensitization), and none assessed chronic toxicity. Doses in rodent studies ranged from 2 to 32,000 mg/kg-day. Only one rodent developmental study, for BPAF, was identified from the published literature[45]. Otherwise all developmental or reproductive studies from the published literature were in fish models testing concentrations of 0.1 to 1,500 µg/L. Additional animal studies including developmental, reproductive and aquatic toxicology endpoints were identified in the ECHA database for BPS, BDP, BPS-MAE, and Pergafast 201 (Table 4).

In Vitro Evidence

One hundred thirty *in vitro* studies reporting on 16 BPA analogues were identified in the published literature. These studies are summarized in Table 5. Although many of the studies evaluated multiple BPA analogues or other chemicals, approximately half (70/130) reported on

the effects of only a single BPA analogue, thus limiting ability to make direct comparisons across studies on the BPA analogues. Eighty-six of the 130 studies evaluated the ability of the BPA analogues to interact with nuclear receptors; for example, interaction with the estrogen receptor was reported in 68 studies and interaction with the androgen receptor was reported in 18 studies). *In vitro* studies of genetic toxicity were also summarized in the ECHA database for BPS, BDP, BPS-MAE, and Pergafast 201 (Table 5).

Health Outcomes and Mechanistic Findings

Repeated Dose Effects

Subchronic effects of repeated exposure (13-28 days) to BPAF, 4,4-BPF, BPZ or BPS were evaluated in five rodent studies in the published literature[43; 59; 76; 138; 154] (Supplemental Figure 1, Supplemental Figure 2) and 23 additional studies in the ECHA database. These studies evaluated changes in body and organ weight as well as other signs of toxicity, such as clinical chemistry. Overall, the most sensitive endpoints include body, kidney, brain, and liver weight (Table 6). In addition to the publicly available literature, there were 28- and 90-day repeated dose studies available in the ECHA database for BPS, BPS-MAE, BDP and Pergafast 201 (Table 6). BDP did not appear to cause any treatment related effects, including on body weight[31; 36], and there were no study details available for the one study reporting subchronic exposure of rats to BPS-MAE[35].

Reproductive and Developmental Effects

Only one rodent developmental study was identified from the published literature. In this study, rats were dosed with BPAF via gavage at 200 to 500 mg/kg-day from gestation day 14 to 18 and testosterone production was evaluated in the male offspring at gestation day 18[45]. Developmental exposure to BPAF did not alter fetal testosterone production in this assay. In addition, there were reproductive and developmental rodent studies identified in the ECHA database for BPS, Pergafast 201, BDP, and BPS-MAE which are summarized in Supplemental Table 12.

Pergafast 201 decreased F1 body weight at the same dose that caused maternal kidney and liver toxicity (200 mg/kg-day). BPS reduced the number of F1 offspring at the same dose that disrupted maternal estrus cyclicity and implantation (300 mg/kg-day). There were no effects reported for BDP, and no information available for BPS-MAE.

Reproductive and developmental effects of exposure to six BPA analogues (4,4-BPF, BPS, BPAF, BPB, BPE, BPAP) were tested in zebrafish models (Table 4)[68; 101; 123; 126; 137; 155]. Developmental exposure to all of these BPA analogues except 4,4-BPF (which was only tested in a single study) caused some degree of morphological malformation ranging from pericardial edema to otic vesicle deformities, for example. Steroidogeneisis and hatching endpoints were disrupted in adult zebrafish exposed to BPS or BPAF[68; 155].

Table 4. Summary of Animal Evidence

	4,4-BPF	BPS	BPAF	BPB	BPC	BPE	BPZ	TMBPA	BPAP	2,2-BPF	BDP	2,4-BPS	BPS-MAE	Pergafast
Number of studies	**15**	**9 [31]**	**10**	**9**	**5**	**3**	**3**	**1**	**2**	**2**	**1 [126]**	**1**	**[21]**	**[14]**
Endpoint classification (number of studies by endpoint)														
Acute toxicity	1^{R},1^{Rb}	1^{R}, 1^{Rb}, 1^{GP} [5^{R}, 2^{M}, 2^{GP}]	1^{R}	–	1^{R}	–	–	–	–	–	[21^{R}, 2^{M}]	1^{R}, 1^{Rb}	[4^{R}]	[3^{R}]
Carcinogenicity	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Reproductive	–	2^{ZF} [1^{R}]	2^{ZF}	–	–	–	–	–	–	–	[2^{R}]	–	[1^{R}]	[1^{R}]
Developmental	1^{ZF}	2^{ZF} [1^{R}]	1^{R}, 3^{ZF}	1^{ZF}	–	1^{ZF}	–	–	1^{ZF}	–	[9^{R}]	–	[2^{R}]	–
Neurological	–	1^{ZF}	–	–	–	–	–	–	–	–		–	–	–
Repeated Dose	1^{R}	1^{R} [3^{R}]	2^{R}, 1^{ZF}	–	–	–	1^{R}	–	–	–	[16^{R}]	–	[1^{R}]	[3^{R}]
Skin Sensitization	1^{GP}	1^{GP} [1^{M}, 2^{GP}]	–	–	–	–	–	–	–	1^{GP}	[14^{GP}]	–	[2^{GP}]	[1^{GP}]
Eye Irritation	1^{Rb}	1^{Rb} [4^{Rb}]	–	–	1^{Rb}	–	–	–	–	–	[13^{Rb}, 1^{P}]	–	[2^{Rb}]	[1^{Rb}]
Dermal Irritation	1^{GP}, 1^{Rb}	1^{GP} [3^{Rb}, 1^{GP}]	–	–	1^{Rb}	–	–	–	–	1^{GP}	[13^{Rb}]	–	[2^{Rb}]	–
Endocrine Activity	8^{R}	1^{R}, 1^{ZF}	3^{R}	7^{R}	1^{R}	–	2^{R}	1^{Fr}	1^{R}	1^{R}	–	–	–	–
Aquatic Toxicity (Acute)	1^{D}	2^{D} [2^{F}, 2^{D}]	–	1^{D}	1^{D}	1^{F}, 1^{D}	–	–	–	–	1^{D} [12^{F}, 14^{D}]	–	[2^{F}, 2^{D}]	[1^{ZF}, 1^{D}]
Aquatic Toxicity (Chronic)	–	[1^{D}]	–	–	–	–	–	–	–	–	[1^{ZF}, 1^{F}, 8^{D}]	–	[1^{F}, 1^{D}]	[1^{F}, 1^{D}]
Other Studies	1^{M}, 1^{GP}	1^{F}	–	–	2^{P}	–	–	–	–	–	–	–	–	–
Doses tested														
Range (mg/kg) in mammalian studies	2-1,000	10-32,000	4-11,000	2-600	25-63	–	6-300	–	not listed	2-200	15-5,000	1,400-10,000	100-2,000	12.5-2,000
Range (µg/L) in aquatic studies	200-14,016	0.1-500,000	5-23,536	242-16,692	–	214-14,998	–	2.84-284	290-20,325	–	21-100,000	–	66-14,000	890-86,000

Superscript indicates species: ^{R}Rat, ^{M}Mouse, ^{GP}Guinea Pig, ^{Rb}Rabbit, ^{P}Poultry, ^{Fr}Frog, ^{ZF}Zebra Fish, ^{F}Other Fish, ^{D}Daphnia.

Numbers in [ ] are from ECHA database and are not included in any of the summary or overall counts. – indicates no studies were found.

Table 5. Summary of *In Vitro* Evidence

	4,4-BPF	BPS	BPAF	BPB	BPC	BPE	BPZ	TMBPA	BPAP	BPP	2,4-BPS	2,2-BPF	BDP	BPPH	D-8	Pergafast 201	BPS-MAE
Number of studies in published literature	**61**	**52**	**41**	**35**	**22**	**23**	**15**	**14**	**8**	**6**	**1**	**1**	**2**	**2**	**2**	**1**	**0**
Endpoint classification (total number for each endpoint)																	
Estrogen Receptor (68)	32	26	29	25	13	13	11	8	5	3	–	1	–	1	2	[1]	–
Androgen Receptor (18)	13	11	5	6	3	4	2	1	–	–	–	–	–	–	1	1	–
Thyroid Receptor (6)	1	1	3	1	3	1	–	4	–	–	–	–	–	–	–	–	–
Cytotoxicity (11)	2	7	1	1	1	1	–	–	–	–	–	–	1	–	1	1	–
Miscellaneous/not otherwise classified(25)	13	11 [1]	1	3	1	3	–	3	1	–	–	–	1	–	1	1	–
Adipocytes (3)	2	2	–	2	–	2	–	–	–	–	–	–	–	–	–	–	–
Pregnane X Receptor (3)	3	3	2	1	–	1	1	1	–	–	–	–	–	–	–	–	–
Constitutive androstane receptor (2)	2	2	1	–	–	–	1	1	–	–	–	–	–	–	–	–	–
Estrogen Related Receptor γ (3)	2	1	3	2	–	2	–	–	1	–	–	–	–	–	–	–	–
Retinoic Acid Receptor γ (2)	2	2	–	2	–	2	1	–	–	–	–	–	–	–	–	–	–
Retinoid-related Orphan Receptor γ (1)	1	1	–	–	1	1	1	–	1	1	–	–	–	1	–	–	–
Glucocorticoid Receptor (3)	2	2	1	–	1	–	1	–	–	1	–	–	–	–	–	–	–
Aryl hydrocarbon Receptor (1)	1	1	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–
Albumin or SHBG Binding (5)	1	2	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Genotoxicity (14)	8	6 [10]	5	–	6	1	1	1	1	1	1	–	[36]	–	–	[3]	[4]

Numbers in [ ] are from ECHA database and are not included in any of the summary or overall counts. – indicates no studies were found.

Table 6. Summary of Most Sensitive Endpoints in Subchronic Studies

Study	Animal Group	Endpoint	Doses Tested (mg/kg/-day)	NOEL (mg/kg-day)	LOEL (mg/kg-day)	Direction of Change (% Change Relative to Control at LOEL)
Published Literature						
4,4-BPF						
Higashihara et al. [59]	Female Crj:CD (Sprague-Dawley) rat	kidney weight	20, 100, 500	20	100	↑ (8.1%)
Higashihara et al. [59]	Female Crj:CD (Sprague-Dawley) rat	body weight	20, 100, 500	–	20	↓ (11.6%)
Higashihara et al. [59]	Female Crj:CD (Sprague-Dawley) rat	brain weight	20, 100, 500	–	20	↑ (14.1%)
BPAF						
Feng et al. [43]	Male Sprague-Dawley rat	body weight	2, 10, 50, 200	10	50	↓ (12.3%)
Umano et al. [138]	Female Sprague-Dawley rat	body weight	10, 30, 100	10	30	↓ (7.3%)
BPZ						
Yamasaki and Okuda [154]	Male CRL:CD (Sprague-Dawley) rat	heart weight	30, 100, 300	30	100	↓ (10.9%)
Yamasaki and Okuda [154]	Male CRL:CD (Sprague-Dawley) rat	serum T4	30, 100, 300	–	30	↑ (14.7%)
ECHA Database						
BPS	Male Wistar rat	kidney weight	100, 300, 1000	–	100	↑ (10%)
BPS-MAE	Female Crj:CD (Sprague Dawley) IGS rat	clinical signs	40, 200, 1000	200	–	Details not provided.
BPS-MAE	Male Crj:CD (Sprague Dawley) IGS rat	kidney weight	40, 200, 1000	40	200	↑ (Details not provided.)
BDP	Male and female Sprague-Dawley rat	–	–	1000	–	Details not provided.
Pergafast 201	Female Wistar rat	liver weight	12.5, 25, 50, 150	–	12.5	↑ (14.7%)

– indicates no studies were found.

Estrogenic and Anti-estrogenic Activity

Seven studies evaluated the effects of BPAF, BPB, 2,2-BPF, 4,4-BPF, BPS, and BPZ on the uterotrophic response and 68 studies assessed estrogenic effects in vitro, mostly for 4,4-BPF (32), BPS (26), BPAF (29), BPB (25), BPC (13), BPE (13), TMBPA (8), and BPAP (6) (Table 5). Like BPA, the BPA analogues were shown to have varying levels of estrogenic activity in the test systems evaluated, but the majority had activity within the same order of magnitude as BPA. However, all chemicals tested were less potent than positive control reference agonists such as E_2.

In vivo estrogenic endpoints evaluated included (a) the uterotrophic response (Figure 2, Supplemental Figure 3, Supplemental Figure 4)[2; 50; 129; 147; 150; 151; 153], (b) the uterine glycogen deposition response[8], (c) assessment of vaginal smears for cornified cells[16; 27; 28; 129] and (d) measurement of vitellogenin[101; 123; 126; 155] or other E_2-responsive genes in fish[68]. The uterotrophic assay is a standard assay in which juvenile or ovariectomized rodents are administered test substance for three to five days after which time the effects on uterine growth are observed. The BPA analogues tested in this assay (BPAF, BPB, 2,2-BPF, 4,4-BPF, BPS, BPZ) were all uterotrophic (Figure 2, Supplemental Figure 3, Supplemental Figure 4). Because the chemicals were not tested over the same dose range it is difficult to assess which chemical is the most potent in this assay. In general, however, 2,2-BPF and BPS appeared to be weaker than the other tested analogues. Another well-characterized assay for determining estrogenicity is the induction of vitellogenin (VTG) gene or protein expression in juvenile or male fish. BPS[101] and BPAF[123; 126; 155] both induced plasma or liver VTG expression (Supplemental Figure 5). In the one study that evaluated both BPA and BPAF, BPAF was more potent (more estrogenic) than BPA at inducing VTG[126].

In vitro estrogenic endpoints were evaluated for fourteen BPA analogues in 68 studies (Table 5). *In vitro* estrogenic endpoints evaluated included (a) receptor binding, (b) modulation of cellular proliferation, (c) modulation of reporter genes transfected into immortalized cell lines, (d) modulation of endogenous estrogen-responsive genes and proteins (e.g. progesterone receptor) or steroidogenesis, (e) the ability to recruit co-regulatory elements, (f) induction of non-genomic signaling pathways, and (g) interaction of ER with ERE. Because of the wealth of *in vitro* estrogenic endpoints, the results of each of these types of assays is discussed in detail in the Supplemental Materials.

The largest number of chemicals were tested in the MCF7 cell proliferation and the reporter gene assays (Table 7). Ten of the BPA analogues demonstrated estrogen receptor agonism in one or more of the assays at concentrations ≤ 1 µM (BPE, BPP, BPAF, BPS, 4,4-BPF, BPC, BPB, BPZ, BPAP, TMBPA). BPAF was consistently one of the most potent BPA analogues in the estrogen agonist and antagonist assays (Figure 2C). Conversely BPS and TMBPA, tended to be some of the least potent BPA analogues (Figure 2C, Table 7). BPE was interesting in that it was one of the most potent BPA analogues in stimulating MCF7 cell proliferation[100] but one of the weakest at binding ER[54]. D8 was not active in any of the estrogen activity assays it was tested in (ER binding, reporter gene, and steroidogenesis assays)[47; 81; 133] but did antagonize E_2 activity at 50 µM[81]. In Tox21 BPAF was the most potent ER agonist of the BPA analogues, and was more potent than BPA. BPZ, BPC, TMBPA, 4,4-BPF, BPB, BPE, BPS displayed ER agonism similar to BPA (AC_{50} between 0.2 and 2 µM). PHBB was weaker (AC_{50} ~20 µM). 2,2-BPF, TCBPA,

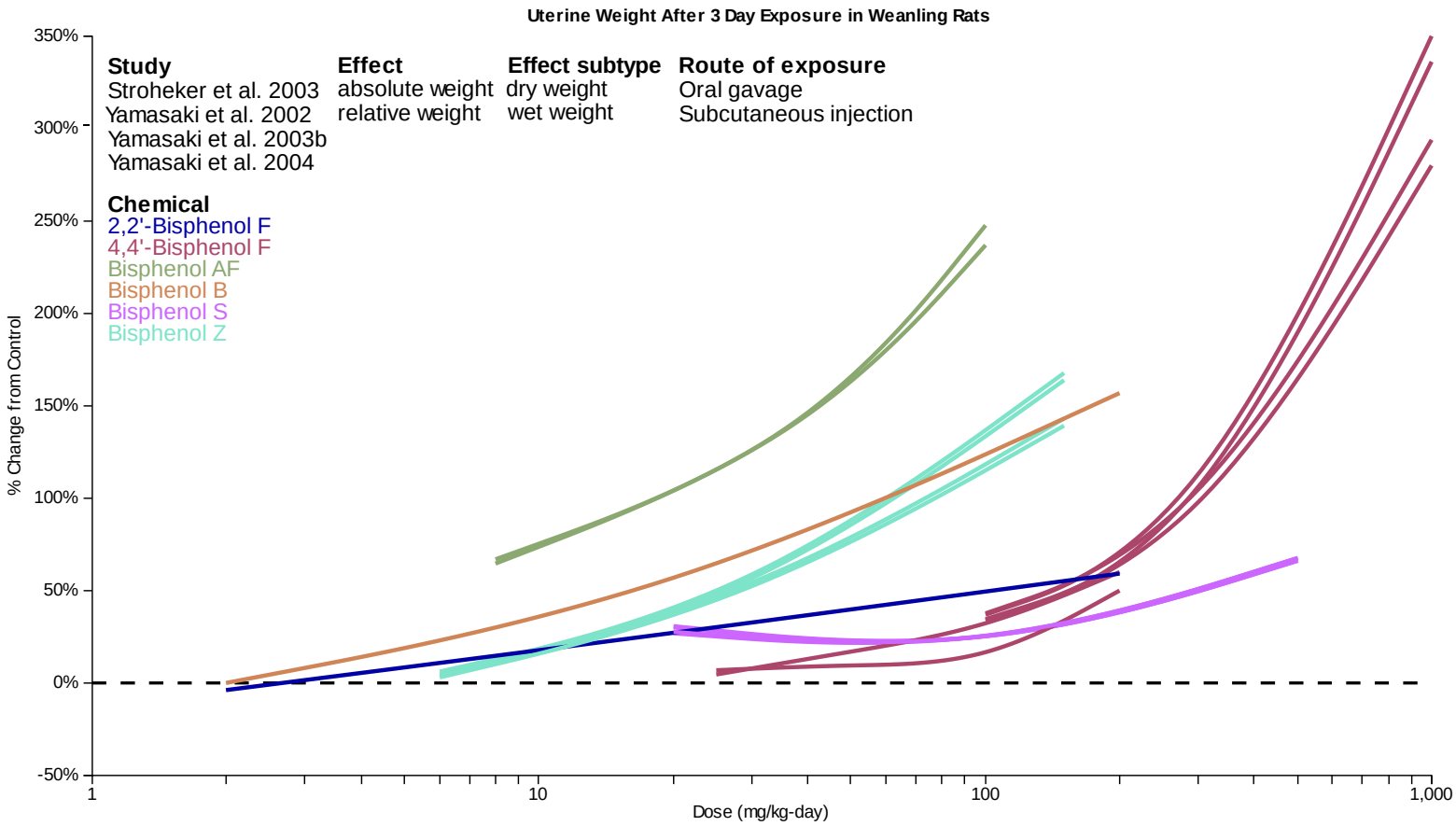
TBBPA only stimulated the partial receptor at ~50 µM. ER antagonism at ~50 µM was observed for TGSA, TCBPA, BPZ, BPC, TMBPA, BPAF, BPE, and BPB.

Table 7. Summary of *In Vitro* Estrogen Endpoints

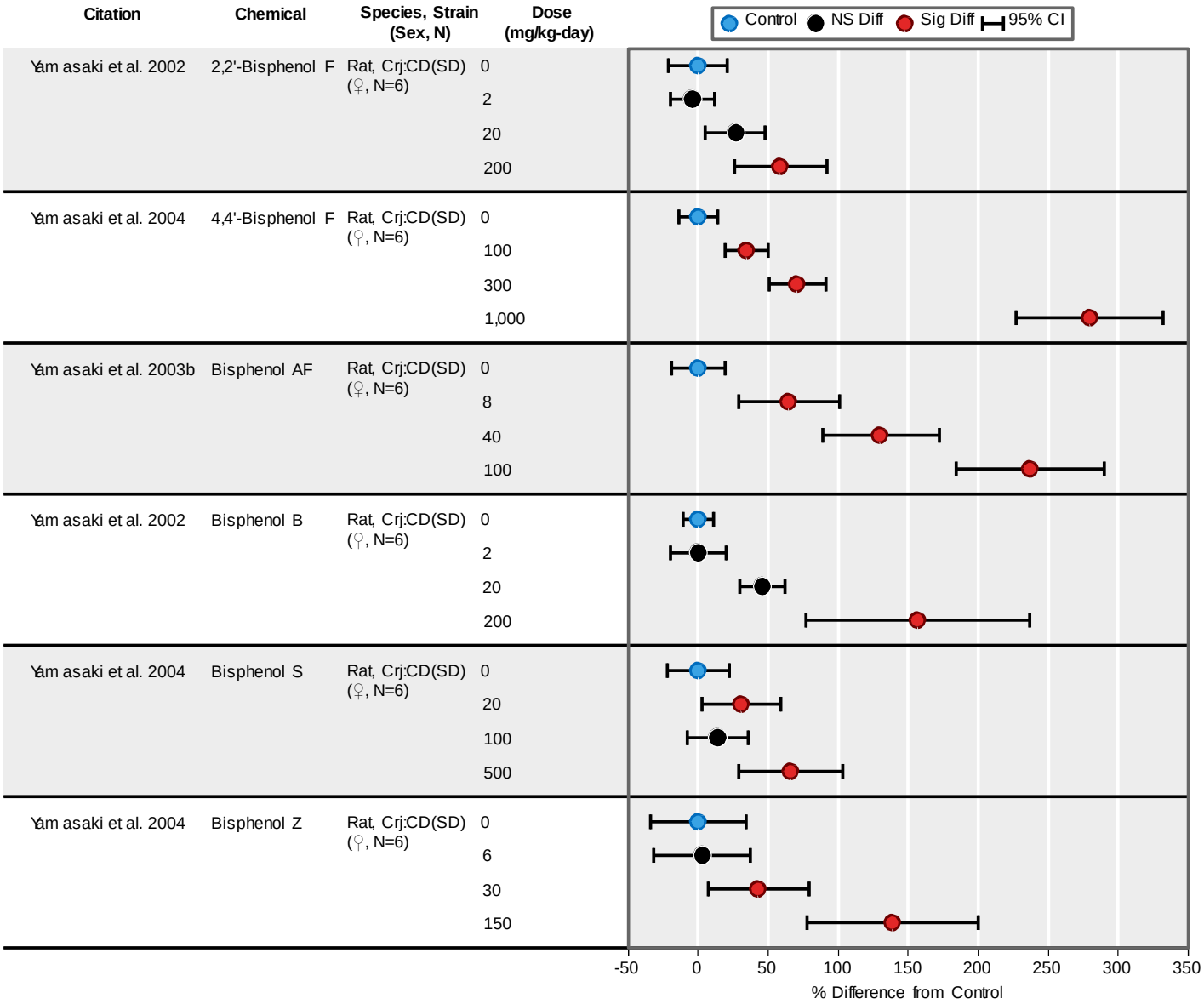
	Lowest Effect Level Reported (µM)						
	ER binding	**MCF7 cell proliferation**	**Reporter gene assay**	**Tox21 ER reporter assay**	**Endogenous gene and protein expression***	**Steroidogenesis**	**ER binding to ERE**
BPE	100	0.00001	0.01		10	3.14	
BPP		0.00001	2.51				
BPAF	0.0003	0.001	0.001		0.01		0.01
BPS	1	0.01	0.0001		10	ND	1
4,4-BPF	0.01	0.01	0.01		1	3.14	0.01
BPC	0.01	0.01	0.04		0.1		1
BPB	0.023	0.01	0.1		0.1	6.25	0.01
BPZ		10	0.0043		10		0.01
BPAP		10	5		10		0.01
BPPH		10					
TMBPA			0.73		1		0.01
D-8	ND		ND			ND	
Pergafast 201						ND	

*most of these tested at only a single dose. ND, not detected.

A


Uterine Weight After 3 Day Exposure in Weanling Rats
Study
Stroheker et al. 2003
Yamasaki et al. 2002
Yamasaki et al. 2003b
Yamasaki et al. 2004
Effect
absolute weight
relative weight
Effect subtype
dry weight
wet weight
Route of exposure
Oral gavage
Subcutaneous injection
Chemical
2,2'-Bisphenol F
4,4'-Bisphenol F
Bisphenol AF
Bisphenol B
Bisphenol S
Bisphenol Z
% Change from Control
350%
300%
250%
200%
150%
100%
50%
0%
-50%
1
10
100
1,000
Dose (mg/kg-day)

B


Citation
Chemical
Species, Strain (Sex, N)
Dose (mg/kg-day)
Control
NS Diff
Sig Diff
95% CI
Yamasaki et al. 2002
2,2'-Bisphenol F
Rat, Crj:CD(SD) (♀, N=6)
0
2
20
200
Yamasaki et al. 2004
4,4'-Bisphenol F
Rat, Crj:CD(SD) (♀, N=6)
0
100
300
1,000
Yamasaki et al. 2003b
Bisphenol AF
Rat, Crj:CD(SD) (♀, N=6)
0
8
40
100
Yamasaki et al. 2002
Bisphenol B
Rat, Crj:CD(SD) (♀, N=6)
0
2
20
200
Yamasaki et al. 2004
Bisphenol S
Rat, Crj:CD(SD) (♀, N=6)
0
20
100
500
Yamasaki et al. 2004
Bisphenol Z
Rat, Crj:CD(SD) (♀, N=6)
0
6
30
150
-50
0
50
100
150
200
250
300
350
% Difference from Control

C

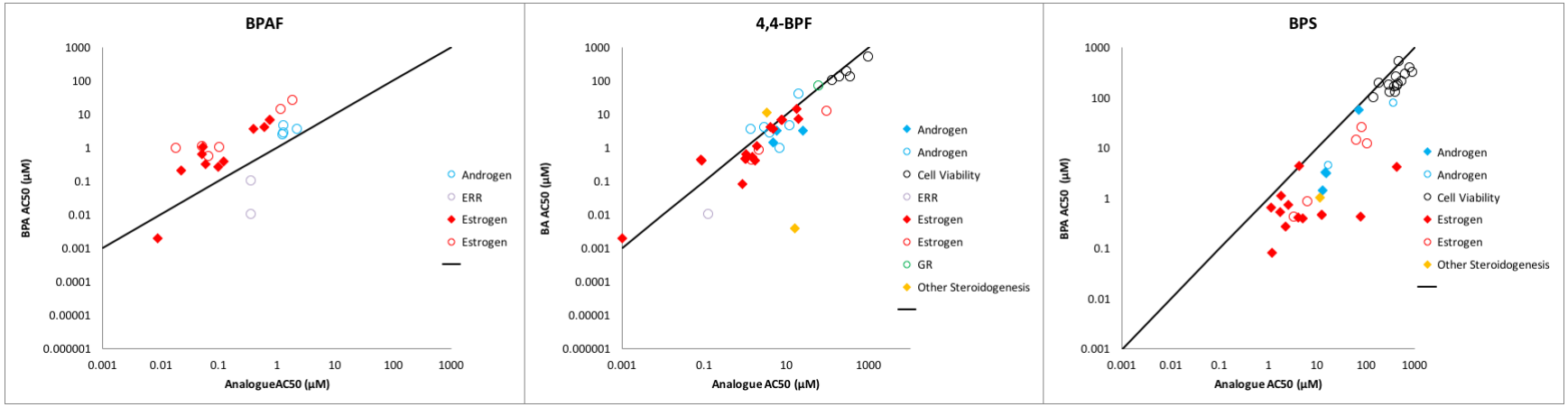


Figure 2. Estrogenic Activity

(A) Summary of uterotrophic responses (dry, absolute uterine weight) following three days of exposure in weanling rats. Live link with all available uterotrophic data available in Supplemental Figure 3; (B) Summary of uterotrophic responses (dry, absolute uterine weight) by dose and % change from control for studies with subcutaneous exposure. Live link available for all routes of exposure and data reporting subtypes (dry or wet weight, relative or absolute) available in Supplemental Figure 4; (C) Scatterplot showing the relationship between reported AC_{50} values (µM) for BPA and BPAF, 4,4-BPF or BPS. Agonist assays are shown with closed diamonds and antagonist assays are shown with open circles. The color of the symbol indicates the type of assay (androgen-blue; estrogen-red; cell toxicity-black, estrogen related receptor (ERR)-purple, glucocorticoid receptor-green; steroidogeneisis of other hormones-orange). When the BPA analogue has an AC_{50} more potent than BPA the points fall above the black line and when the BPA analogue has an AC_{50} less potent than BPA the points fall below the black line.

Androgenic and Anti-androgenic Activity

In vivo endpoints to assess androgen-regulated outcomes included (a) the Hershberger assay[151], (b) disruption of testis function in rats exposed as adults[43] or developmentally[45], and (c) male reproductive organ weights after subchronic exposure[32; 34; 37; 43; 59; 138]. The lack of effects following developmental and subchronic exposures to BPAF, 4,4-BPF, BPZ, BPS, BPS-MAE, BDP, or Pergafast 201 have already been discussed above[31-34; 36; 37; 43; 45; 59; 138; 154]. In the Hershberger assay adult male Brl Han:WIST Jcl rats were exposed for 10 days to 50, 200 or 600 mg/kg-day BPAF, BPB or 4,4-BPF via oral gavage[151]. There were few effects reported from the Hershberger assay except that exposure to BPB (200 and 600 mg/kg-day) and BPAF (200 mg/kg-day) decreased the relative bulbo cavernous/levator ani muscle weight and BPAF (600 mg/kg-day) increased relative glans penis weight (Supplemental Figure 13).

Both androgen agonism (Supplemental Figure 14) and antagonism (Supplemental Figure 15) were assessed *in vitro.* Androgen endpoints *in vitro* included (a) receptor binding, (b) reporter gene activity, (c) cellular proliferation, and (d) steroidogenic production of testosterone, androstenedione, or dehydroepiandrosterone. Androgen receptor (AR) binding was only explored for two analogues, BPB and BPS; BPB was found to bind AR with similar potency to BPA[39; 61] whereas BPS only bound AR weakly[39]. Eight analogues (BPAF, BPB, BPC, BPE, 4,4-BPF, BPS, BPZ, TMBPA) were tested for AR agonist activity (Supplemental Figure 14). There was very limited evidence of weak agonism for two analogues (4,4-BPF, and BPS), but this was not replicated in other studies of the same chemicals. All eight analogues were, however, anti-androgenic in the range of 0.01 to 100 µM (Supplemental Figure 15). TMBPA and BPAF were the most potent anti-androgens, with TMBPA ten times more potent that the positive control anti-androgen flutamide[75]. Of the anti-androgenic analogues, BPS appeared to be the weakest, acting as an anti-androgen in some assays but not others. Two additional BPA analogues, D-8 and Pergafast 201, were also tested for AR activity in a single study in which neither analogue altered androgen steroidogenesis[47] (Supplemental Figure 11). In Tox21, none of the analogues were AR agonists. Like BPA, TBBPA, BPAF, BPB, BPZ, BPE, 4,4-BPF, 2,2-BPF, BPC, TGSA, TMBPA were AR antagonists in the Tox21 assays between 3 and 100 µM. BPS, TCBPA, PHBB, were inactive in Tox21 AR antagonist assays.

Thyroid and Anti-thyroid Activity

In vivo studies of thyroid disruption were identified for 4,4-BPF, BPAF, BPS, TMBPA, and BPZ and included evaluation of thyroid hormone levels in rats[59; 138; 154] or fish[101; 155], and disruption of frog metamorphosis[49] (Supplemental Figure 16). Subchronic (28 day) studies were performed in young adult rats in which increased plasma T4 was observed in males exposed to 4,4-BPF (500 mg/kg-day)[59], BPAF (100 mg/kg-day)[138], or BPZ (30 mg/kg-day)[154] and in females exposed to 4,4-BPF (20 mg/kg-day) and BPAF (100 mg/kg-day). In male rats treated with 500 mg/kg-day 4,4-BPF the T4 increase was accompanied by a decrease in plasma T3 and an increase in relative thyroid weight[59]. In adult zebrafish treatment for 75 days with BPS or 28 days with BPAF had seemingly opposite effects: exposure to BPS reduced plasma T3 and T4 in males (10 and 100 µg/L) and females (100 µg/L), whereas exposure to BPAF increased whole body homogenate levels of free T3 in females at 1000 µg/L but had no effect in males[101; 155]. In developing frogs TMBPA treatment (28.4 and 284 µg/L) for 9 days suppressed tail regression and spontaneous metamorphosis[48].

In vitro studies of thyroid hormone receptor activity were identified for 4,4-BPF, BPS, BPAF, BPB, BPC, BPE, and TMBPA[12; 13; 72; 74; 75; 124]. Endpoints used to assess thyroid hormone activity *in vitro* included (a) TR binding, (b) TR reporter gene activity in yeast, (c) growth hormone (GH) production in GH3 pituitary cells, (d) cellular proliferation of GH3 cells, and (e) inhibition of deiodinase or sulfotransferase activity in liver microsomes as a measure of receptor antagonism (Supplemental Figure 17, Supplemental Figure 18). Receptor binding was only assessed for TMBPA and BPC, both of which bound TR within the range of 1-100 µM[74]. TMBPA induced TRα and β mediated reporter gene activity between the doses of 1 and 10 µM[124] and GH production in GH3 cells[75], but did not induce GH3 cell proliferation[72]. The opposite effects were noted for BPC, which stimulated GH3 cell proliferation[72], but did not induce GH production[75]. There was no evidence that BPAF, BPB, BPE, 4,4-BPF, or BPS were thyroid hormone agonists as none of these chemicals stimulated GH production in GH3 cells[75]. BPAF, however, disrupted thyroid hormone homeostasis by inhibiting deiodinase and sulfotransferase activity in liver microsomes[12; 13]. In the Tox21 assays there is no evidence that the BPA analogues were TR agonists or antagonists with the exception of TCBPA, which appeared to be a TR antagonist but this was confounded by concurrent cytotoxicity.

Other Receptor Activity

The ability of BPA analogues to interact with additional nuclear receptors (PXR, CAR, ERRγ, RARγ, RORγ, GR, and AhR) was also explored in the *in vitro* studies we identified (Supplemental Figure 19). There were three or fewer *in* vitro studies identified for each of these nuclear receptors. Of these, only ERRγ was also evaluated *in vivo*, where three of five tested BPA analogues disrupted otolith development in zebrafish[137]. *In vitro* 4,4-BPF, BPAF, BPB, and BPE bound ERRγ, induced the expression of a reporter gene, and antagonized the activity of the ERRγ inverse-agonist 4-hydroxytamoxifen[26; 107]. Several of the analogues were found to be agonists for PXR and one or more CAR isoform (4,4-BPF, BPAF, BPB, BPE, BPZ, TMBPA)[29; 131], which was consistent with the results from Tox21 that indicated these and BPC to be agonists and BPZ and BPAF to be CAR antagonists. However, some of these findings were not repeated with endogenously expressed receptors[66; 111]. TMBPA, BPAF, BPC BPB, were found to be GR antagonists in Tox21. Though there was relatively little information on GR activity in the published literature it appeared that 4,4-BPF competitively bound GR but BPP and BPS did not[78; 117]. 4,4-BPF, BPB, BPE, and BPZ did not bind RARγ, nor did they or BPAF, BPAP, BPC, and BPS bind RORγ[69; 102]. In contrast, in the Tox21 analysis, BPAF, BPC, BPB, TGSA were identified as RORγ antagonists.

Acute Toxicity and DNA Damage

Acute oral and dermal toxicity studies reported the lethality in 50% of the exposed animals (LD_{50}) following a single exposure. The studies identified from the database search all appeared to be company documents submitted to US EPA and contained very few study details[3; 30; 76; 99]. Of these studies, BPC had the lowest LD_{50} of 25 mg/kg in orally exposed mice. The oral LD_{50} for BPAF, 2,4-BPS, and BPS ranged from 3,600 to >7,000 mg/kg. Several studies of acute oral and dermal toxicity for BPS, Pergafast 201, BDP and BPS-MAE were also identified in the ECHA database where the LD_{50} was in the rage of 1,600 to >5,000 mg/kg.

In vitro analyses of DNA damage and toxicity were identified in the published literature as well as in the ECHA database and included (a) Ames test, (b) CHO/HGPRT mutation assay, (c)

chromosomal aberration assays, (d) Comet assay, (e) micronucleus assay, (f) cellular proliferation, (g) cell transformation assay, and (h) expression of p53 or γH2AX (Supplemental Figure 20). The tests use different cell types, different methods with or without metabolic activation, and not all reported if concurrent cytotoxicity was an issue. As noted in the figure, a large number of the studies found some increase in genotoxicity compared to vehicle treatment conditions. There were also some decreases in measures of genotoxicity that could not always be explained by increases in cytotoxicity. 4,4-BPF was the most studied (Table 5) with increases in genotoxicity noted in the Ames assay with and without metabolic activation and in cell transformation studies. Similar results were also observed with BPAF, but more consistent results were observed for increased chromosomal aberration. Genotoxicity was consistently increased by BPC as observed in the cell transformation, chromosomal aberration, micronuclei assays, or the Ames assay with or without metabolic activation. The other analogues tested for genotoxicity (BPAP, BPB, BPE, BPP, BPS, BPZ, and TMBPA) either had conflicting results or there were not enough of the same tests to form conclusions (Supplemental Figure 20), but there was a suggestion of increased genotoxicity for each of these. In Tox21, BPAF and BPB induced p53 between 50 and 60 µM. In most cases the mitochondrial toxicity assay was the most sensitive assay for cell stress in Tox21. For the most part the AC_{50} for mitochondrial toxicity was ≥10 µM, except for TCBPA and BPAF, in which the AC_{50} was <5 µM. There were no indications of DNA damage or cell stress after BPS exposure in the Tox21 assays.

Other Endpoints

A handful of additional endpoints have been explored for the BPA analogues. Binding to alpha fetoprotein or sex hormone binding globulin has been evaluated for BPB, 4,4-PBF, and BPAF[61; 109; 148]. *In vitro* neurotoxicity was assessed for BDP and BPAF[58; 83]. A few studies evaluated the lipid metabolism and adipogenic activity of BPS, BPB, 4,4-BPF, and BPE[57; 95; 111]. Along with various other phenolic compounds, 4,4-BPF has been extracted from the Chinese medical herb *Gastrodia elata Blume* and has been evaluated as a smooth muscle relaxant[56; 161] an anti-inflammatory[82], and a platelet anti-aggregating agent[112]. Other miscellaneous effects explored include: the effects of BPS on rat hearts and isolated myocytes[46], inhibition of the hypoxic response of human hepatoma cells[80], and stimulation of cells to release alkali metal cations [62].

Structural and Biological Similarity Analysis

The analyses of BPA analogues in chemical structure and HTS space is unique because not only do they allow for more analogues to be directly compared to one another than in any of the published reports, but they can also provide a broader perspective of how similar the chemicals are when considered in the context of a larger chemical set such as the entire Tox21 library (>8,000 chemicals).

Structural Similarity Analysis

The structural similarity to BPA or E_2 was assessed for the 25 BPA analogues that were tested in Tox21 (Figure 3). Two BPA analogues, D-90 and UU, were not considered in this analysis because they are polymers. Using chemical structural features to evaluate the relationship between E_2 and the BPA analogues shows a clear separation between E_2 and the BPA analogues with BPZ being the most structurally similar to E_2 (i.e., had the highest Tanimoto Coefficient). The BPA analogues are more structurally similar to BPA, with BPB, 4,4-BPF and BPE being

very similar to BPA and TGSA and PHBB being most dissimilar. A complete list of Tanimoto similarity metrics between BPA and Tox21 library can be found in Supplemental Table 13 and Supplemental Table 14 (column E).

Biological Similarity Analysis Based on HTS Data

Ten BPA analogues were tested either in ToxCast Phase II or the E1K library. These chemicals were tested in a range of 316 to 882 different medium or high throughput assays. Analysis of these chemicals in ToxCast, which covers a wider biological space than Tox21, included assays on cell cycle disruption, DNA binding, growth factors, cell adhesion molecules, cytokines, and proteases that were not assessed in Tox21. The BPA analogues were listed as active in anywhere from 4% to 30% of tested ToxCast assays, most typically in assays for nuclear receptors, DNA binding, GPCR, cell cycle, and cytokines (Supplemental Figure 21). Subsequent assessments of biological similarity consider only the Tox21 data because more chemicals were tested in this platform. Figure 4 shows the half-maximal concentration (AC_{50}) for each BPA analogue in the Tox21 assays. The ER agonism assay was the most sensitive assay for 8 of the 13 BPA analogues tested in Tox21. The BPA analogues were similar to BPA in that they tended to be 100 to 10,000 fold weaker than E_2 in stimulating ER activity. Based on evaluation of the dendogram in Figure 4**,** BPB had the most similar activity to BPA across the Tox21 assays, followed by BPC and BPAF. TGSA,which was not an ER agonist, had the most dissimilar activity compared to BPA, and was the only BPA analogue to activate the aryl hydrocarbon receptor (AhR). These findings are highlighted by the similarity of the ToxPI profiles for compounds within a groups and the difference in ToxPI profiles between the groups (Figure 4).

The biological activity of the 13 BPA analogues was compared to that of 14 pharmacological E_2 analogues. In thirteen assays, including two AR antagonist assays, at least half of both the BPA analogue and E_2 analogue sets had decreasing activity (indicating antagonism or cytotoxicity). In three assays, including two ER agonist assays, at least half of both analogue sets had increasing (agonist) activity. For 10 of the Tox21 assay channels, including PPAR antagonist and aromatase inhibition assays, the BPA analogues set tended to have decreasing activity, while the E_2 analogue set was not consistently active. BPA analogues activated Nrf2 signaling whereas the pharmacological E_2 analogues did not. A complete listing of results of the analysis can be found in the Supplemental Materials and Supplemental Table 10.

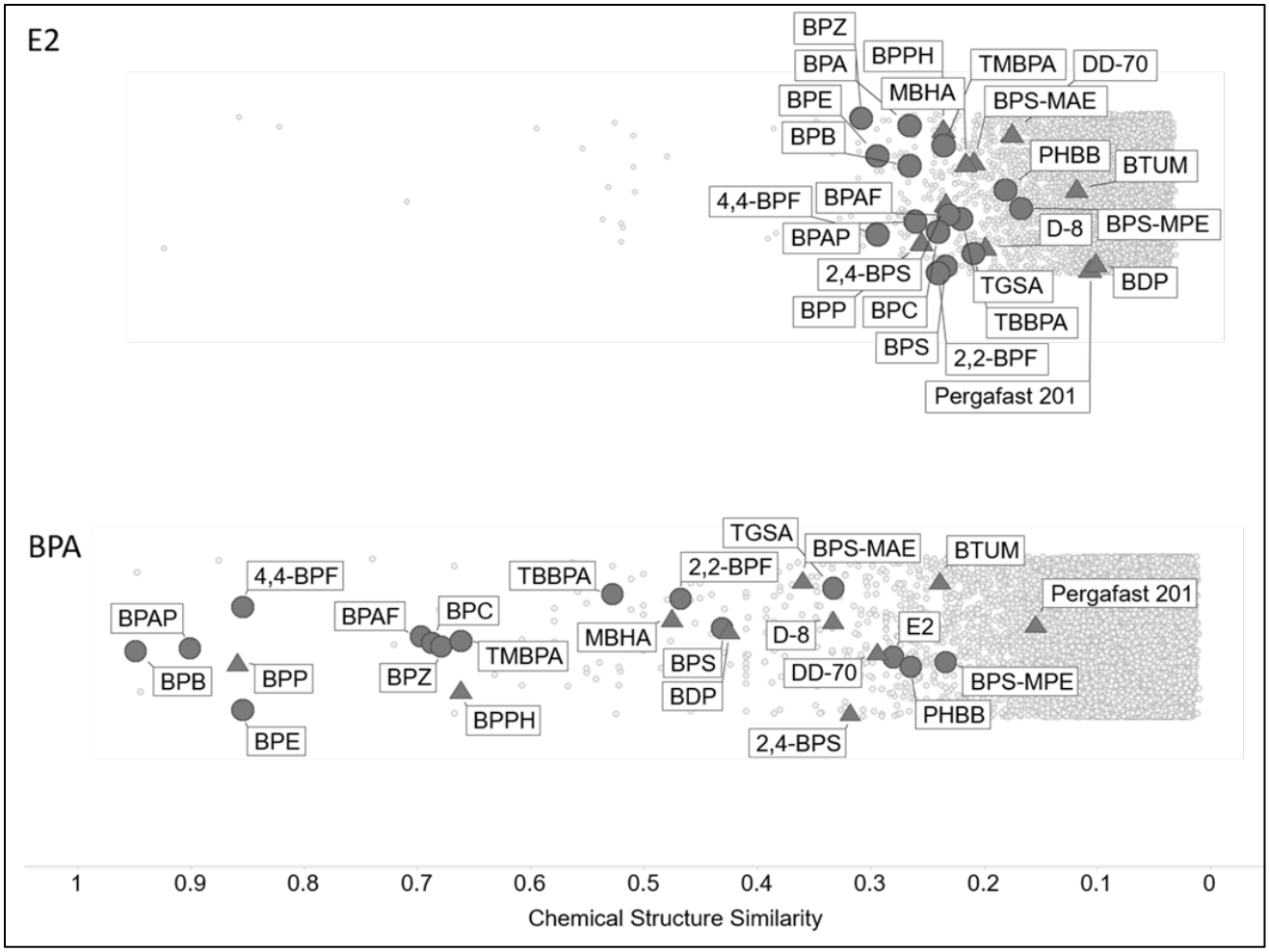


Figure 3. Structural Similarity of BPA Analogues to BPA and E_2

Structural similarity of BPA analogues to (A) BPA or (B) E_2. The horizontal axis is the Tanimoto coefficient (reverse scale; decreasing similarity left to right), a measure of similarity based on the presence or absence of structure features (Leadscope Enterprise v3.4) relative to the reference chemical. A larger coefficient indicates greater structural similarity. Dark circles indicate BPA analogues present in the Tox21 collection (note: BPPH, BPAP and BPS-MPE were only run in limited number of Tox21 assays), triangles are BPA analogues absent from the collection (2,4-BPS, BPS-MAE, BPP, BPPH, DD-70, D-8, BTUM, MBHA, Pergafast 201, BDP), squares are BPA analogues present in the Tox21 collection, but not considered in elsewhere in this review (TBBPA, TCBPA, PHBB), and small circles are 8,278 non-BPA analogue chemicals from the Tox21 collection. Points are jittered in the vertical direction for clarity.

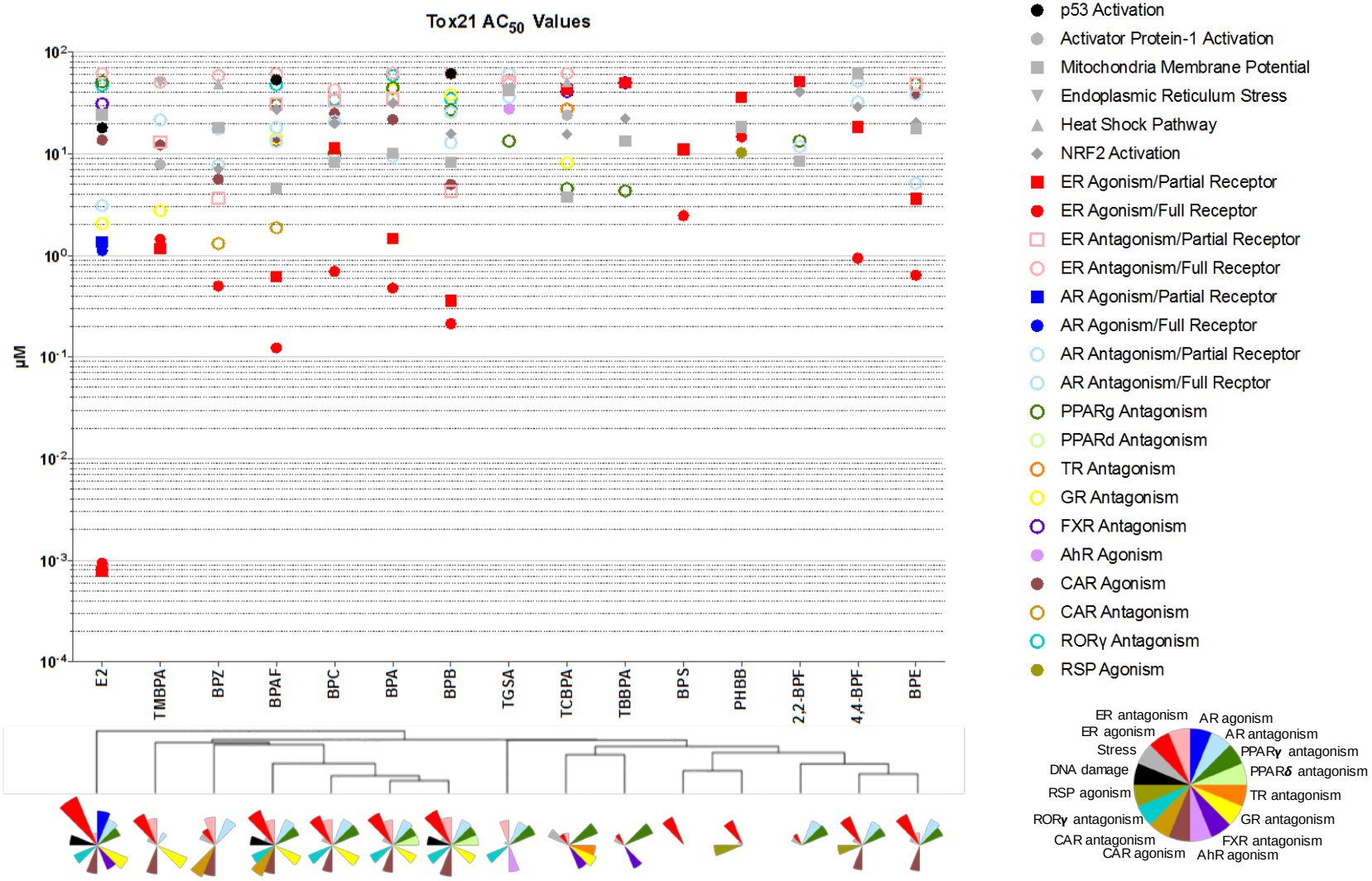


Figure 4. Biological Similarity Analyses in Tox21

Biological similarity of BPA analogues evaluated in Tox21 HTS library. Plot and ToxPI depiction of selected assays based on half maximal concentrations (AC_{50}). The 24 assays in which one or more BPA analogue was active are each represented by a different symbol in the plot.

Chemicals are sorted on the x-axis based on their activity similarity. The dendogram beneath the scatterplot displays the Euclidean distance based on respective activities and a hierarchical clustering with average linkage. Raw data are available in Supplemental Table 8. In the ToxPI depiction of biological similarity the height of the pie slice for each assay category indicates how active that chemical is for that type of assay (*i.e.* – log(AC_{50})) relative to the most potent chemical-assay pair within the library (i.e. dioxin and AhR agonism). The dendogram was generated based on biological similarity across the assays shown. Abbreviations are as follows: Nuclear factor erythroid 2-related factor 2 (Nrf2), estrogen receptor (ER), androgen receptor (AR), farnesoid X receptor (Fxr), Aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor (PPAR), thyroid receptor (TR), glucocorticoid receptor (GR), constitutive androstane receptor (CAR) retinoid-related orphan receptor (ROR) retinol signaling pathway (RSP).

Another biological similarity analysis determined the relative similarity of 13 Tox21 BPA analogues (including TCBPA, TBBPA, and PHBB) in Tox21 to BPA and E_2 in the context of the entire Tox21 library (Figure 5). This was done by determining the Pearson correlation coefficient between E_2 or BPA and the entire Tox21 library. E_2 appeared to be most similar to BPB and TMBPA. The least similar analogues to E_2 were BPPH and BPAP, neither of which were significantly associated with E_2. Overall the BPA analogues exhibit much greater biological similarity to BPA than the average chemical in the Tox21 library. Like the above analysis, BPA appeared to be most similar to BPB, BPC, and BPE. The least similar analogues to BPA were BPS, BPAP and BPPH. All analogues, even those least similar to BPA, exhibited highly significant association with BPA in this analysis. Supplemental Table 13 and Supplemental Table 14 provide a complete listing of BPA and E_2 correlation values, respectively, for the entire Tox21 library along with confidence intervals and an association statistic. Note: the values in the supplementary table are listed by substance (i.e., not averaged by CASRN) hence the same CASRN may have multiple correlation values.

Integrated Chemical and Biological Similarity

In vitro assay results such as those from Tox21 and chemical structure can provide complementary information on the biological effects of chemicals. We present in Figure 6 a plot that integrates the biological (from Figure 5) and chemical structure similarity (from Figure 3) of the BPA analogues relative to E_2 and BPA. This was done in the context of the chemicals from the Tox21 library, hence several of the analogues were not included, due to lack of or limited HTS data (2,4-BPS, BPPH, BPS-MAE, BPP, DD-70, BPS-MPE, D8, BPAP, BTUM, MBHA, Pergafast 201, and BDP) or lack of both HTS and structure information (D-90, UU). Chemicals shown at the upper right of the graph have the highest degree of combined similarity to BPA or E_2 (Figure 6). In the case of E_2 the BPA analogues tend to cluster in the middle to lower left of the graph, albeit still showing some distinction from the overall set of Tox21 compounds. BPAP is not shown on the E_2 plots because its biological correlation with E_2 was less than 0.25. With BPA, the chemicals in the upper right of the graph include BPB, BPE, BPF, BPAF, and BPC. An overall assessment of the analysis suggests most of the BPA analogues exhibit a relatively high degree of combined similarity to BPA as they are all shifted to the upper right relative compared to the average chemical in the Tox21 library, suggesting that most of the BPA analogues are likely to share biological effects with BPA. This finding is less robust with E_2, although the association with E_2 is still significant (see Supplemental Table 14), therefore suggesting a marginal degree of similarity may exist between E_2 and the BPA analogues.

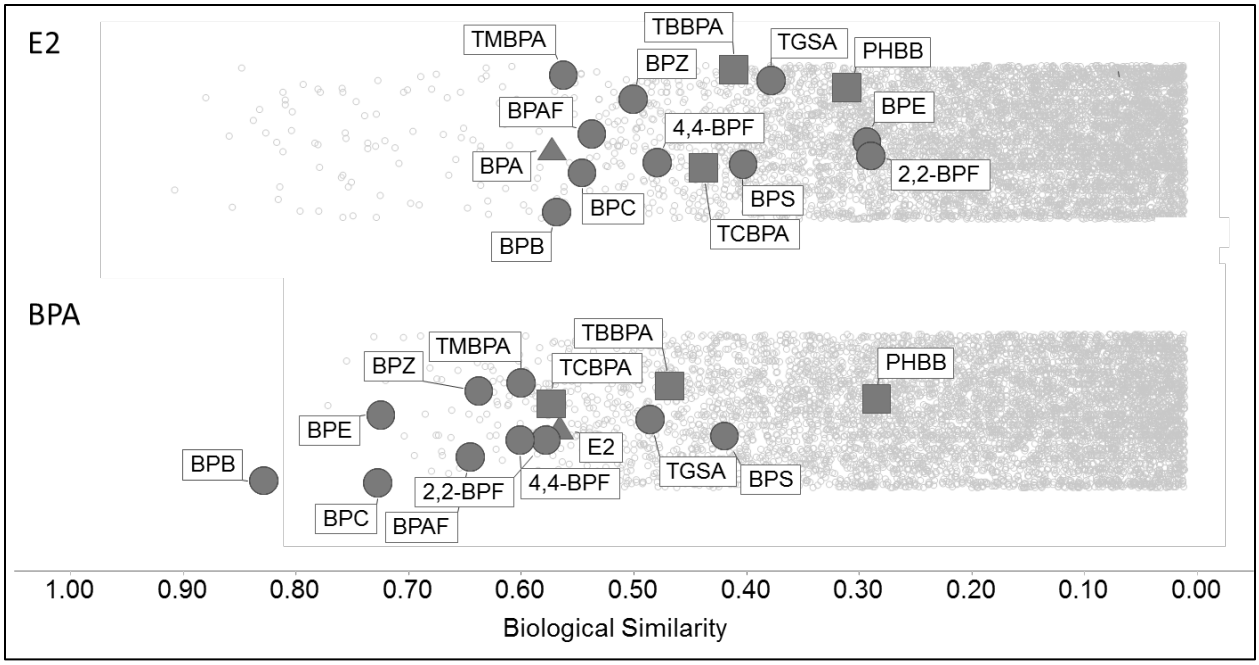


Figure 5. Biological Similarity of BPA Analogues to E_2 and BPA in Context of the Entire Tox21 Library

Biological similarity of BPA analogues to BPA and E_2 in context of the entire Tox21 Library. The horizontal axis is the Pearson Correlation Coefficient (reverse scale; decreasing similarity left to right), computed from Tox21 HTS Assay profiles for BPA analogues each compared to BPA or E_2. A larger coefficient (i.e. close to 1) indicates greater biological similarity to BPA. Dark circles are BPA analogues, squares are BPA analogues in the Tox21 library that are not considered elsewhere in this review (TBBPA, TCBPA PHBB), triangles are reference chemicals, and small circles are 6,053 non-BPA analogues from the Tox21 collection. Points are jittered in the vertical direction for clarity. Some chemicals are present in the chemical library more than once. For these chemicals the Pearson Correlation Coefficients were averaged to acquire a single correlation value per CASRN identifier pair. No BPA analogues were negatively correlated. BPPH, BPAP and BPS-MPE were included in the Tox21 library, however they were only screened in a limited number of assays and are therefore not included in the analysis.

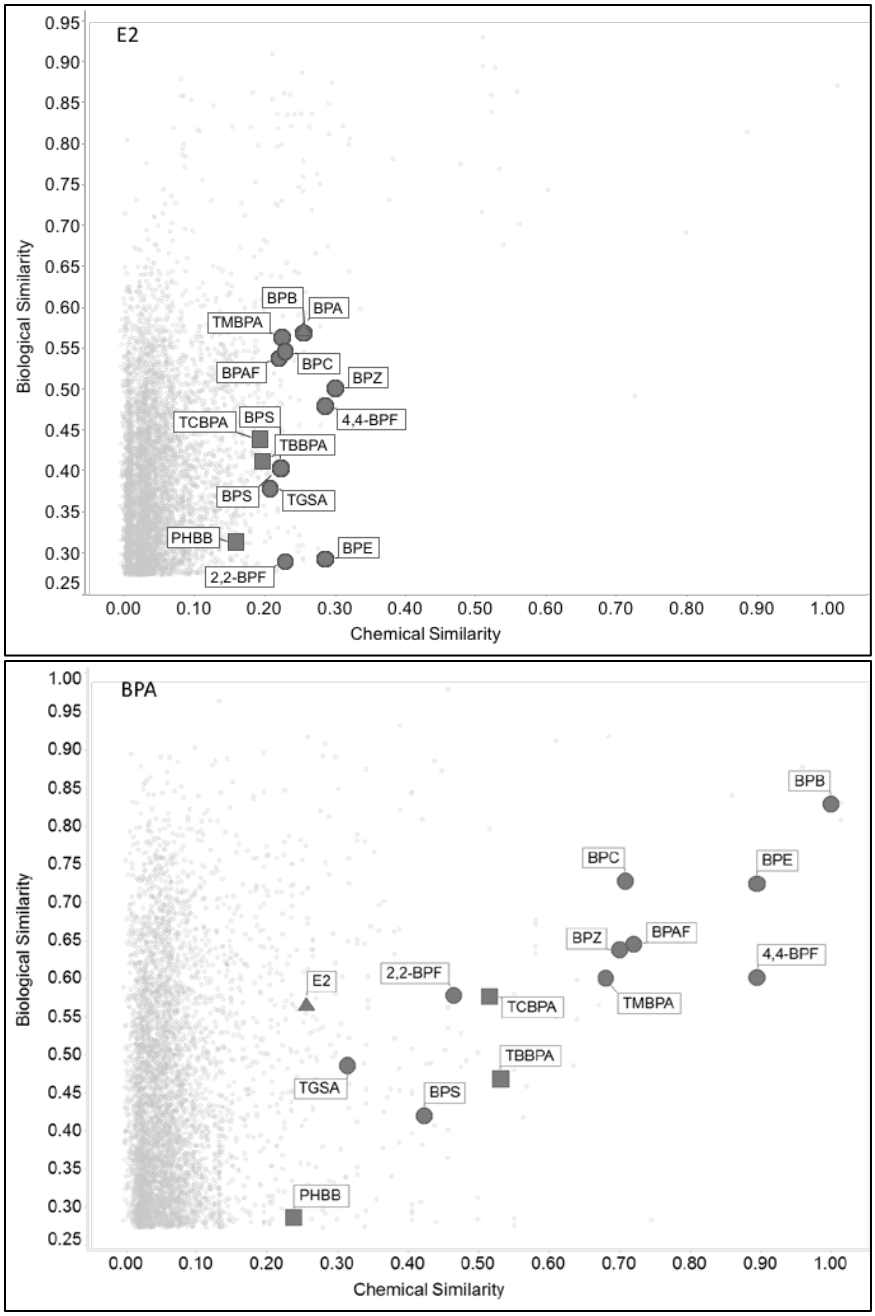


Figure 6. Integrated Structural and Biological Similarity of the BPA Analogues to E_2 and BPA

Integrated structural and biological similarity of the BPA analogues to BPA or E_2. Pearson correlation coefficients to BPA or E_2 of chemicals represented by multiple samples in the chemical library were averaged to acquire a single correlation value per CASRN identifier pair. BPA analogues that were not included in the Tox21 library could not be included in the graph because biological similarity could not be determined. Dark circles are BPA analogues, squares are BPA analogues in the Tox21 library that are not considered elsewhere in this review (TBBPA, TCBPA PHBB), triangles are reference chemicals, and small circles are 6,053 non-BPA analogues from the Tox21 collection. BPPH, BPAP and BPS-MPE were included in the Tox21 library, however they were only screened in a limited number of assays and are therefore not included in the analysis.

Discussion

In this review we sought to identify, synthesize and summarize the existing evidence based on associated health outcome or biological response for 24 BPA structural or functional analogues. The systematic literature search identified 166 relevant studies as well as data from the publicly available databases for the high throughput screening platforms Tox21 and ToxCast and the ECHA database. From the published literature and database searches we identified one or more studies for 17 of the BPA analogues and no studies for seven of the chemicals. The majority of the available data were *in vitro* (n=130 studies), but there were also a significant amount of animal (n=39 studies) and a few human studies (n=4). The *in vitro* work focused largely on the ability of the BPA analogues to act as endocrine disrupting chemicals by binding to, activating or antagonizing various steroid receptors. Of the BPA analogues with one or more study available, there is growing evidence suggesting that most are of high concern. Eight of the 16 chemicals reviewed here and in the DfE received either a high or very high hazard ID in one or more category of human health effect or ecotoxicity (4,4-BPF, BPC, BPS, BPS-MAE, BPS-MPE, D8, Pergafast 201, and TGSA), indicating that these chemicals are of high concern[140]. However, these conclusions were not always based on empirical, publicly available data. The current review adds additional information and makes greater use of available *in vitro* data by presenting the biological and chemical similarities of the BPA analogues. When considered together, these data highlight that BPAF, BPAP, BPB, BPE, BPP, BPZ, and TMBPA are also of concern given their ability to affect endocrine related endpoints at concentrations below 1 µM. For the remaining BPA analogues, there is an overall lack of empirical evidence from which to draw conclusions. In some cases, the hazard classifications seem to range from low to moderate (UU, D-90) based on confidential studies submitted to US EPA[140]. For others (BPPH, 2,4-BPS, DD-70, MBHA, BTUM) there are estimates of high ecotoxicity hazard but empirical evidence is lacking[140]. Importantly, a lack of evidence does not indicate a lack of effect, rather it indicates that more research is needed to fully characterize the biological activity of the data poor BPA analogues.

Data Gaps and Research Needs

For some BPA analogues (4,4-BPF, BPAF, BPAP, BPB, BPC, BPE, BPP, BPPH, BPS, BPZ, Pergafast 201, TMBPA) there is sufficient evidence to suggest the potential for endocrine disruption. Despite this, we noted several limitations to the evidence base:

- Some chemicals are very data poor. For seven chemicals (BPS-MPE, BTUM, D-90, DD-70, MBHA, TGSA, UU) there were no studies either in the published literature or publicly available databases. Only 10 of the 24 chemicals were investigated in three or more studies.
- While not the focus of this review, it is apparent that there is a need for more exposure assessment. This information would help prioritize which data poor chemicals should be assessed in additional toxicological testing. As it is the lack of exposure information makes it difficult to prioritize the data poor chemicals for subsequent testing, even when there is concern for potential hazard.

- For three chemicals (BPS-MAE, BDP, and Pergafast 201) the majority of available data was found in the ECHA database. While most of the studies included in the ECHA database are conducted according to guideline study procedures, the reports in the ECHA database are generally lacking in presentation of study details, including methods and effect sizes.
- Risk of bias was assessed for the animal studies identified from the published literature (Supplemental Figure 22) and from the ECHA database (Supplemental Figure 23). Overall there was a general lack of reporting of key study features including randomization to study groups, allocation concealment, and blinding research personnel throughout the study and at outcome assessment. Future studies should include appropriate considerations in study design, conduct, and reporting in order to minimize bias for the exposure and outcomes considered. At a minimum, studies should include randomization of treatment and blinding of outcome assessors.
- It is important to fully characterize the nature of the dose response of each chemical on the various biological endpoints. The majority of *in vitro* and *in vivo* studies utilized more than one dose of the chemicals. The exception, however, was the assessment of gene expression which was most often performed with only a single treatment level. For a few chemicals the only evidence for a given effect is based on a test of a single dose (e.g. MCF7 cell proliferation after treatment with BPP or BPZ). We strongly encourage researchers to perform a full dose response for all main effects tested in research studies. In this regard, since many of the endpoints examined pertain to disruption of the endocrine system, it is important that researchers be aware of the possibility for non-monotonic response curves and include low, environmentally relevant doses.
- Along similar lines, it was not always clear from some of the *in vitro* studies exactly what doses were tested. In some instances, only an AC_{50} was presented with no indication of the range of doses tested [e.g. Zhang et al. [158]] In other instances, results were presented qualitatively (+ or – activity) [e.g. Kolle et al. [77]] or relative to a positive control [e.g. Coleman et al. [21]], which makes comparison of results across studies difficult. Characterization with and without metabolic activation was primarily only evident in studies of genotoxicity. While some *in vitro* studies utilized cell lines that display limited metabolic competency, others did not. Given how metabolism can dramatically alter the activity of these and similar chemicals future research would benefit from further exploring this question.

Limitations of the Review

This review used systematic review methodology to search for and extract the available data for 24 BPA analogues as of March 2015. As stated in the objectives, the aim of this review was to extract and compile all of the available evidence for this set of chemicals. Given the number of chemicals investigated, the diversity of the various biological effects that were reported, and the lack of an available tool for assessing risk of bias of *in vitro* studies, providing evidence synthesis conclusions was not an aim of this review. Rather, this review has highlighted the

extent and nature or dearth of information available for these 24 BPA analogues. While this review was being conducted, BPA structural and functional analogues have continued to receive much needed research attention. Therefore, a further limitation of this review is the difficulty in incorporating the rapidly growing literature in this field. As such, we are aware of several more recent studies that have not been included in this data synthesis [for example Catanese and Vandenberg [17]; Chen et al. [18]; Kataria et al. [70]; Wang et al. [146]].

Summary

Our results add to a growing literature indicating that risk characterizations of BPA need to expand and should begin to consider BPA structural and functional analogues[135]. This is especially important given that the evidence presented in the current review highlights that many of the BPA analogues are active at concentrations similar to or lower than BPA. Given that many of the BPA analogues are already known to be in use (because they are found in consumer products, house dust, or in biomonitoring specimens), it is important that we increase our knowledge about their potential biological activities. We hope that this systematic review of the literature can serve as a starting point for considering the class of BPA analogues more broadly. It is our hope that future analyses can integrate information from data-rich chemicals such as BPA, BPAF, and BPS to inform scientist about the predicted biological activity of the data poor analogues and thereby avoid situations of regrettable substitution.

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Supplemental Materials

Comparison of Pharmaceutical Estrogen Profiles and BPA Analogues

The activity of the BPA analogues was compared to that of a set of Tox21 chemicals similar to E_2. The E_2 set was defined as all of the Tox21 chemicals with Tanimoto similarity to beta-estradiol of greater than 0.5. The chemicals in this set are given in Supplemental Table 9. The two chemical sets were compared using the direction adjusted, weighted area under curve (wAUC) values for the Tox21 assays. Assay values used were all triplicated assay endpoints, except for the two channels of data used for deriving the ratio in assays where the ratio of two channels was reported.

For each chemical and assay, the median of all replicate wAUC values for that chemical in that assay was obtained. Some chemicals were tested more than once (i.e. have more than one set of triplicate wAUCS), and those values were pooled before calculating the median. For each chemical set and each assay, the assay was assigned an activity sign of 1, indicating consistent increasing wAUC, if more than half of the chemicals (not counting chemicals not tested in that assay) had median wAUC greater than 0 for that assay, a sign of -1 if more than half had median wAUC less than 0, and a sign of 0 otherwise.

Results are shown in Supplemental Table 10. For sixteen of the assays, both the BPA analogues and the E_2 analogues had the same non-zero sign (sign 1 for three assays, sign -1 for 13 assays), designated Group 1 Assays. (This discussion omits assays with sign 0 for both chemical sets). For 10 of the assays designated Group 2 Assays, the BPA analogues set had a sign of -1 while the E_2 analogue set had a sign of 0. Five other assays designated Group 3 Assays had different signs for the two chemical sets.

Hazard Summaries Based on Analogy and Estimation from the US EPA DfE Report

For eight analogues (BPS-MAE, BPS-MPE, BTUM, D-90, DD-70, MBHA, TGSA, UU), no published studies were identified by the literature search or in the US EPA DfE report [140]. In the US EPA DfE report hazard summaries of these chemicals were subsequently derived based on analogy, estimation, or evaluation of data in the ECHA database or submitted confidential studies (Supplemental Table 11). All of these analogues were found or estimated to have a low hazard for acute toxicity, and were estimated as a moderate hazard for carcinogenicity. MBHA achieved a high hazard label for developmental effects based on analogy to BPA and DD-70 achieved a high hazard label for eye irritation based on analogy to a confidential chemical. In contrast to the generally low hazard for human health effects of these analogues, most of them were found or estimated to be moderate, high, or very high hazards for acute or chronic exposure to aquatic organisms. The exceptions were UU and D-90, which were both estimated to be low aquatic toxicity hazards.

Estrogenic and Anti-estrogenic Endpoints

Binding to the estrogen receptor was assessed for ten BPA analogues (4,4-BPF, BPAF, BPB, BPC, BPE, BPS, BPZ, BPAP, TMBPA, D-8) using a number of different assays (Supplemental Figure 6). In some assays binding to the ER was performed in a cell-free context[9; 21; 53; 54; 73; 110; 133]. In others, intact cell systems were utilized to evaluate binding to endogenous ER[130] or exogenously expressed ER in mammalian cells[26; 98] or bacterial cells[2; 96; 152; 153]. Among the strongest binders to ER were BPAF, BPB, BPZ, and BPAP, binding between 0.01 and 1 µM, which was four to ten-fold more potent than that reported for BPA. Binding of BPC, 4,4-BPF, BPE, and TMBPA to ER was similar to that of BPA (ranged from >0.01 to <100 µM), and BPS was the weakest with binding six to 10-fold less potent than BPA (>3 µM). D-8 did not bind to ER in the presence or absence of the metabolic activator S9 fraction[133]. Several BPA analogues bound ERβ more potently than ERα, including BPAF and BPS [96; 98].

Induction of cellular proliferation of human breast cancer cell lines (e.g. MCF7 and T47D) is a well-known characteristic of estrogenic chemicals, and was assessed for 11 BPA analogues (4,4-BPF, BPAF, BPAP, BPB, BPC, BPE, BPP, BPPH, BPS, BPZ, TMBPA). In MCF7 cells the BPA analogues tested were similar to or slightly weaker than BPA in their ability to induce cellular proliferation (Supplemental Figure 7), with the exception of BPAF, which was generally reported as equal to or more potent than BPA[21; 26; 54; 110; 121]. BPS was also interesting in that it was reported as weaker than BPA in inducing MCF7 cell proliferation[53; 54; 98], but was much more potent than BPA in inducing proliferation of the rat pituitary cancer cell line GH3/B6/F10[142]. The pattern of potency was similar for ER binding and cellular proliferation for the chemicals that were tested in both assays with the exception of BPE which was one of the most potent at inducing cellular proliferation[100] but one of the weakest at binding ER[54].

Modulation of reporter genes was assessed in eukaryotic cells transfected with various reporter constructs and with either ERα or ERβ in cells that do not endogenously express the receptor (Supplemental Figure 8). The yeast estrogen screen (YES), in which both the ER and reporter are transformed into yeast cells, was also used. With the exception of D8, all of the BPA analogues tested in these assays were partial to full agonists capable of eliciting induction of the reporter gene similarly to E_2. The majority of the reported EC_{50} values were in the order of 0.1-10 µM for 4,4-BPF, BPB, BPC, BPE, BPP, and TMBPA. BPAF and BPZ were more potent, with EC_{50} values near 0.01-1 µM and BPAP and BPS were less potent with EC_{50} values in the order of 1-10 µM. The potency of the effect was dependent on cell type and receptor (ERα or ERβ). Antagonism was evaluated for 4,4-BPF, BPB, BPC, BPE, BPS, BPAF, and TMBPA. Only BPAF and TMBPA were found to antagonize the activity of E_2, but the reports of BPAF antagonism were conflicting and suggest cell type specific effects[75]. For example potent antagonism of BPAF on ERβ and weak antagonism on ERα was observed in HeLa cells, but not in HepG2, Ishikawa, or MCF-7 cells[26; 75; 87; 132].

In Tox21 BPAF was the most potent ER agonist of the BPA analogues, and was more potent than BPA. BPZ, BPC, TMBPA, 4,4-BPF, BPB, BPE, BPS displayed ER agonism similar to BPA (AC_{50} between 0.2 and 2 µM). PHBB was weaker (AC_{50} ~20 µM). 2,2-BPF, TCBPA, TBBPA only stimulated the partial receptor at ~50 µM. ER antagonism at ~50 µM was observed for TGSA, TCBPA, BPZ, BPC, TMBPA, BPAF, BPE, and BPB.

Modulation of endogenous gene (Supplemental Figure 9) and protein (Supplemental Figure 10) expression was assessed in eukaryotic cells transfected with ERα or ERβ or in cells that endogenously express ER. For the most part only single doses were evaluated in the gene expression studies. All of the tested BPA analogues (4,4-BPAF, BPAF, BPAP, BPB, BPC, BPE, BPS, BPZ, and TMBPA) induced the expression of endogenous estrogen-responsive genes (e.g. prolactin (PRL), growth regulation by estrogen in breast cancer 1 (GREB1), and progesterone receptor (PR)). Though difficult to discern due to the lack of dose response data, BPS appeared the least potent, only inducing gene expression at 300 µM[94] whereas the other analogues induced expression at 1 or 10 µM (Supplemental Figure 9). In general, when ERβ was present there was no induction of endogenous estrogen-responsive genes[128]. In the case of BPAF, however, gene expression was decreased relative to control when cells were transfected with ERβ and increased with ERα[85]. In addition, 4,4-BPAF, BPAF, BPAP, BPB, BPC, BPS, BPZ, and TMBPA were able to antagonize E_2 induced expression of PRL in HeLA cells transfected with ERα or ERβ and GREB1 in MCF7 cells[128].

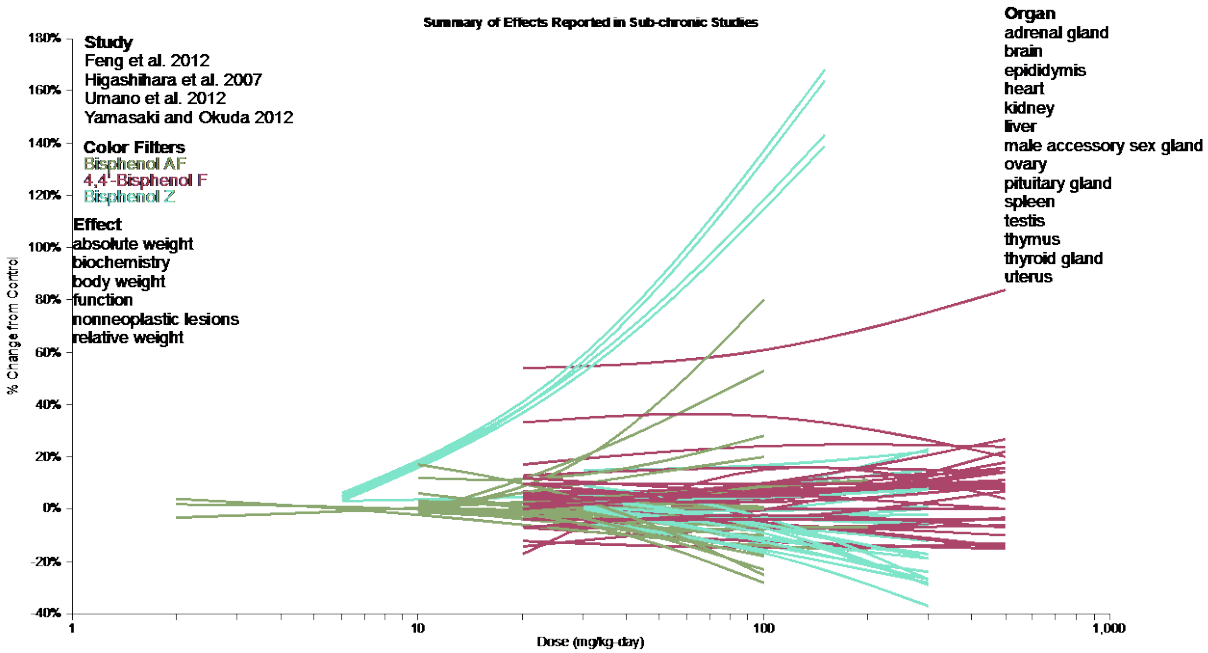
The same chemicals were evaluated for modulation of ERα, PR, PRL, trefoil factor 1(pS2), or VTG protein expression (Supplemental Figure 10). All of the chemicals tested (4,4-BPF, BPAF, BPB, BPC, BPE) increased expression of PR and pS2 in the dose range of 1-10 µM[110; 114]. Whereas BPAF, BPP, BPC, and TMBPA antagonized E_2 induced expression of VTG, only BPAF and BPC acted as agonists to induce VTG expression in the absence of E_2[84]. Stossi et al. [128] reported that 4,4-BPF, BPAF, BPAP, BPB, BPC, BPS, and TMBPA could down regulate ERα levels in MCF7 cells[128].

The production of endogenous estrogens (17β-E_2 and estrone) was assessed for 4,4-BPF, BPB, BPE, BPS, D8 and Pergafast 201 in the human adrenocortical carcinoma cell line, H295R[47; 120] (Supplemental Figure 11). 4,4-BPF, BPB and BPE induced the production of 17β-E_2 and estrone with potencies similar to BPA, whereas BPS was not active in the H295R steroidogenesis assay[47; 120]. For D8 and Pergafast 201 only the production of 17β-E_2 was evaluated [47] and both chemicals were inactive.

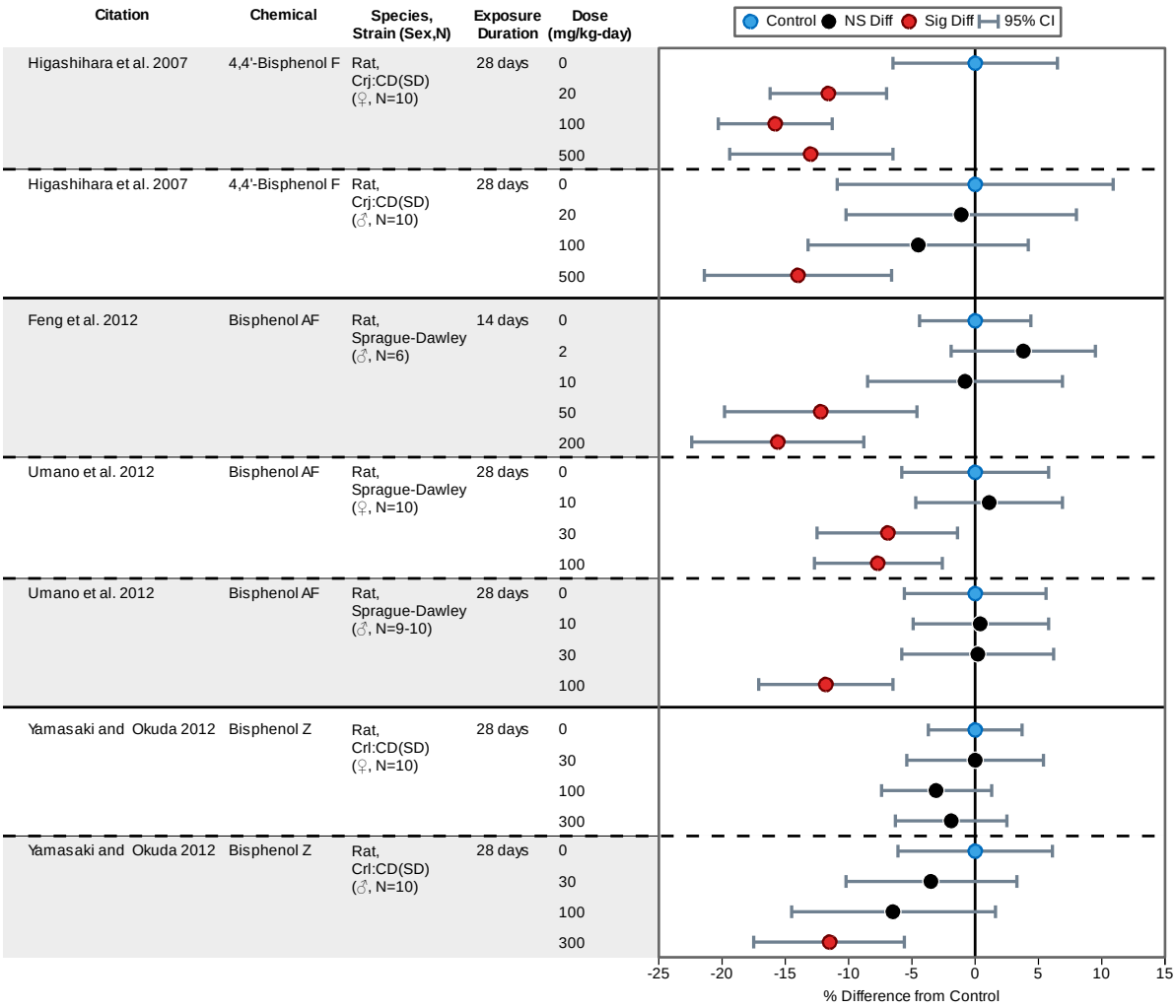
There were additional estrogen receptor related endpoints that were assessed (Supplemental Figure 12). Ashcroft et al. [5] and Stossi et al. [128] evaluated whether or not the BPA analogues could induce ER binding to the estrogen response element (ERE) of DNA using high content imaging. All of the BPA analogues tested (4,4-BPF, BPAF, BPAP, BPB, BPC, BPS, BPZ, TMBPA) demonstrated clear selectivity for ERβ compared to ERα[128] with BPS and 4,4-BPF not able to induce ERα/ERE binding between 0.001 and 10 µM. In the same study, BPAP and BPAF were able to induce ERα or ERβ homodimerization but BPS showed only weak activity[128]. In similar studies Ashcroft et al. [5] demonstrated that BPB could induce ERα nuclear localization and polymerase II recruitment. Non-genomic effects were only explored for BPS, which was able to activate the extracellular signal-regulated kinase ERK and caspase 8 but not JNK or caspase 9[142].

List of Supplemental Tables and Figures

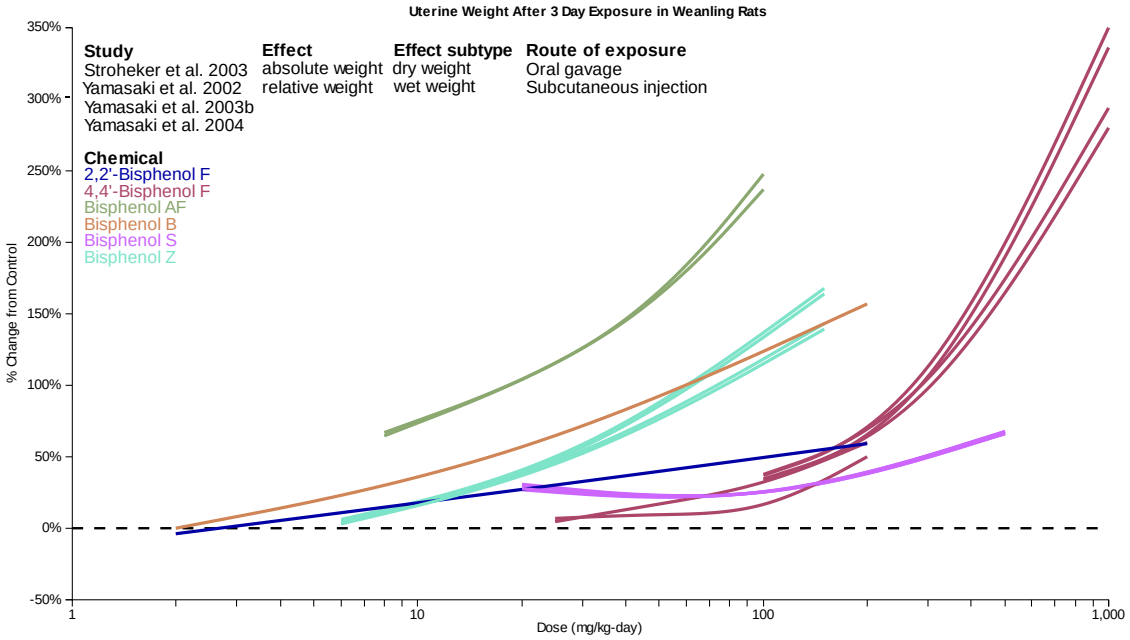
All supplemental tables are available at https://doi.org/10.22427/NTP-DATA-4. Interactive figures and study details are available in HAWC. Each figure label is a clickable hyperlink.



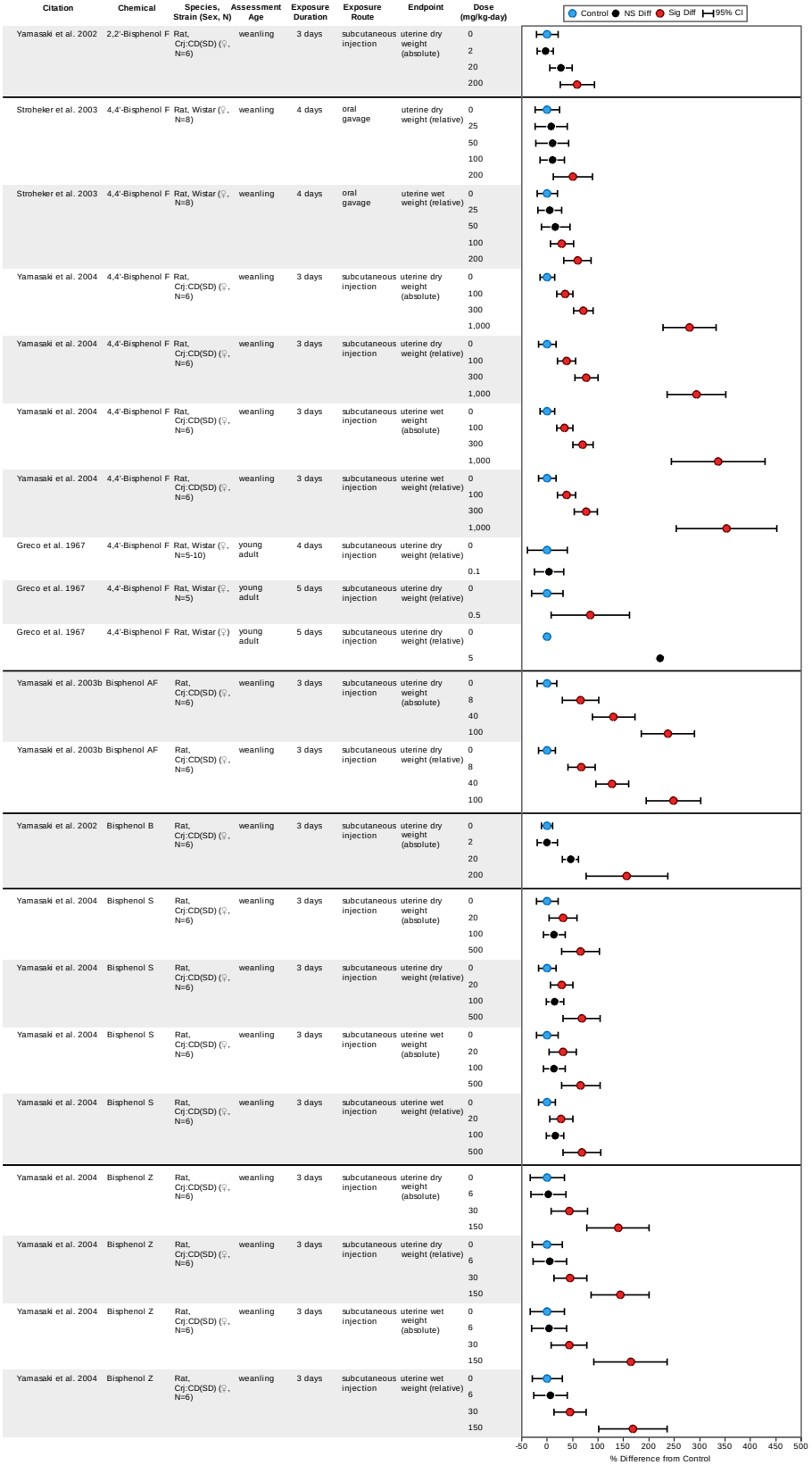
Supplemental Figure 1. Crossview of Subchronic Studies



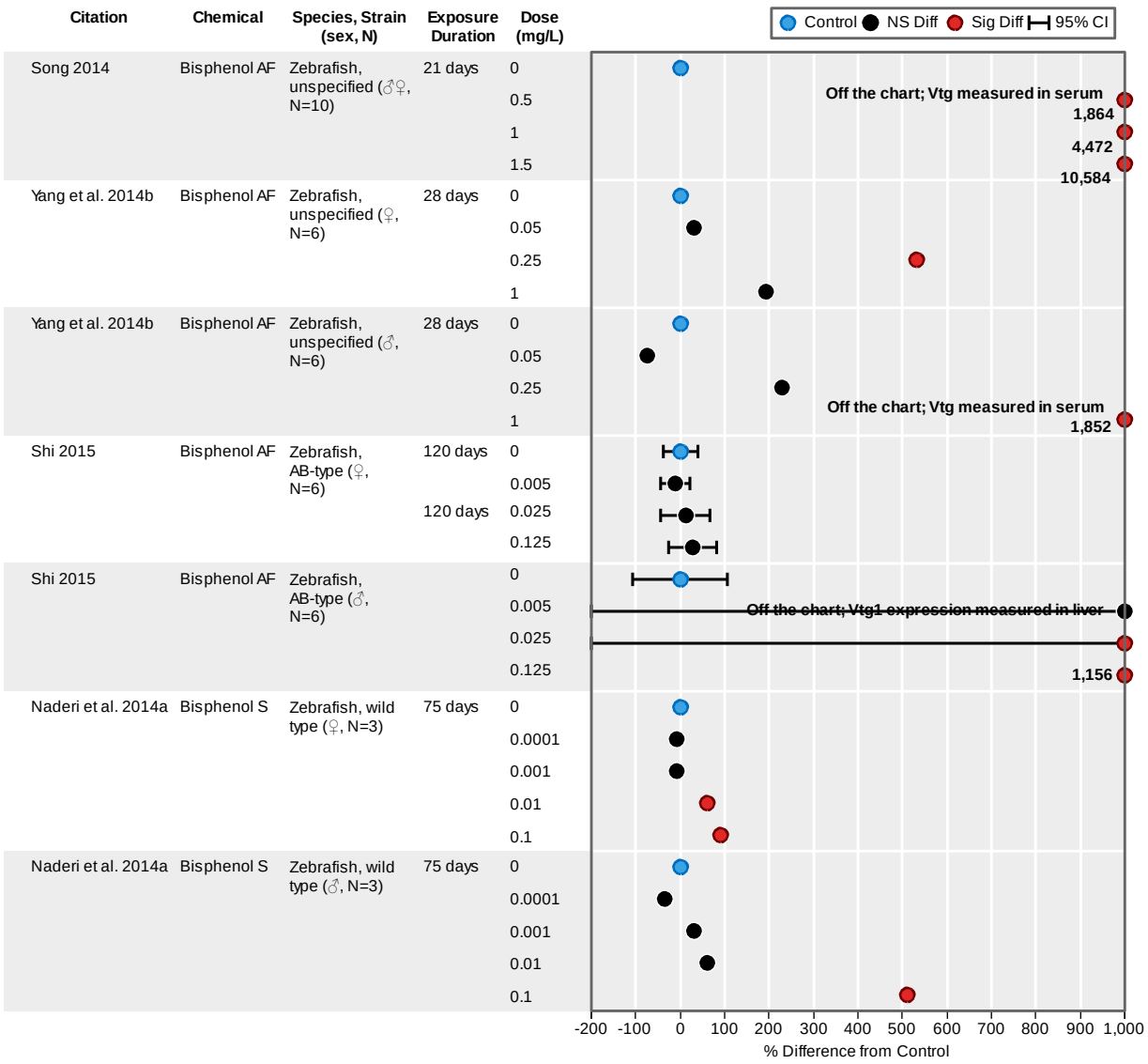
Supplemental Figure 2. Body Weight in Subchronic Studies



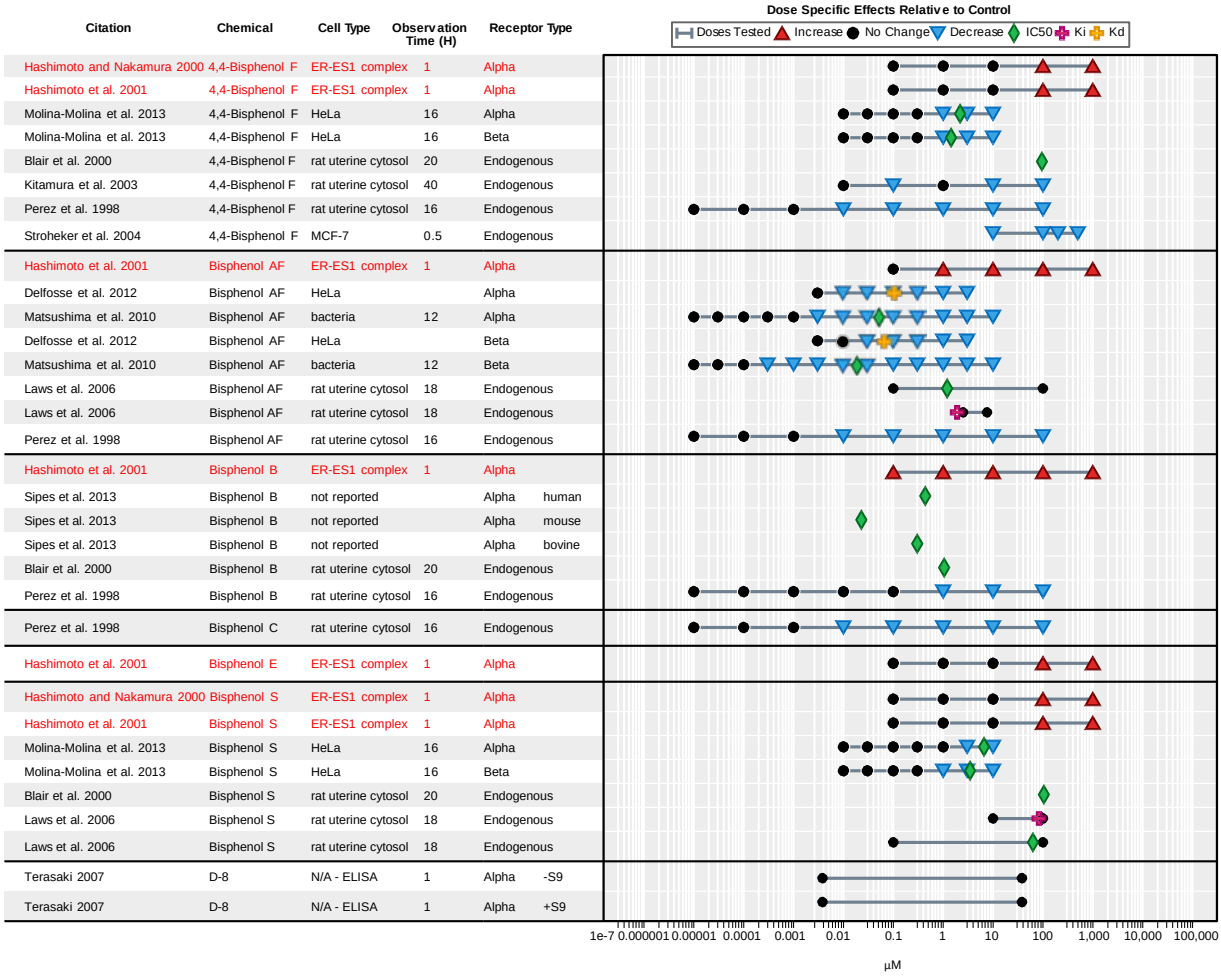
Supplemental Figure 3. Crossview of Uterotrophic Response



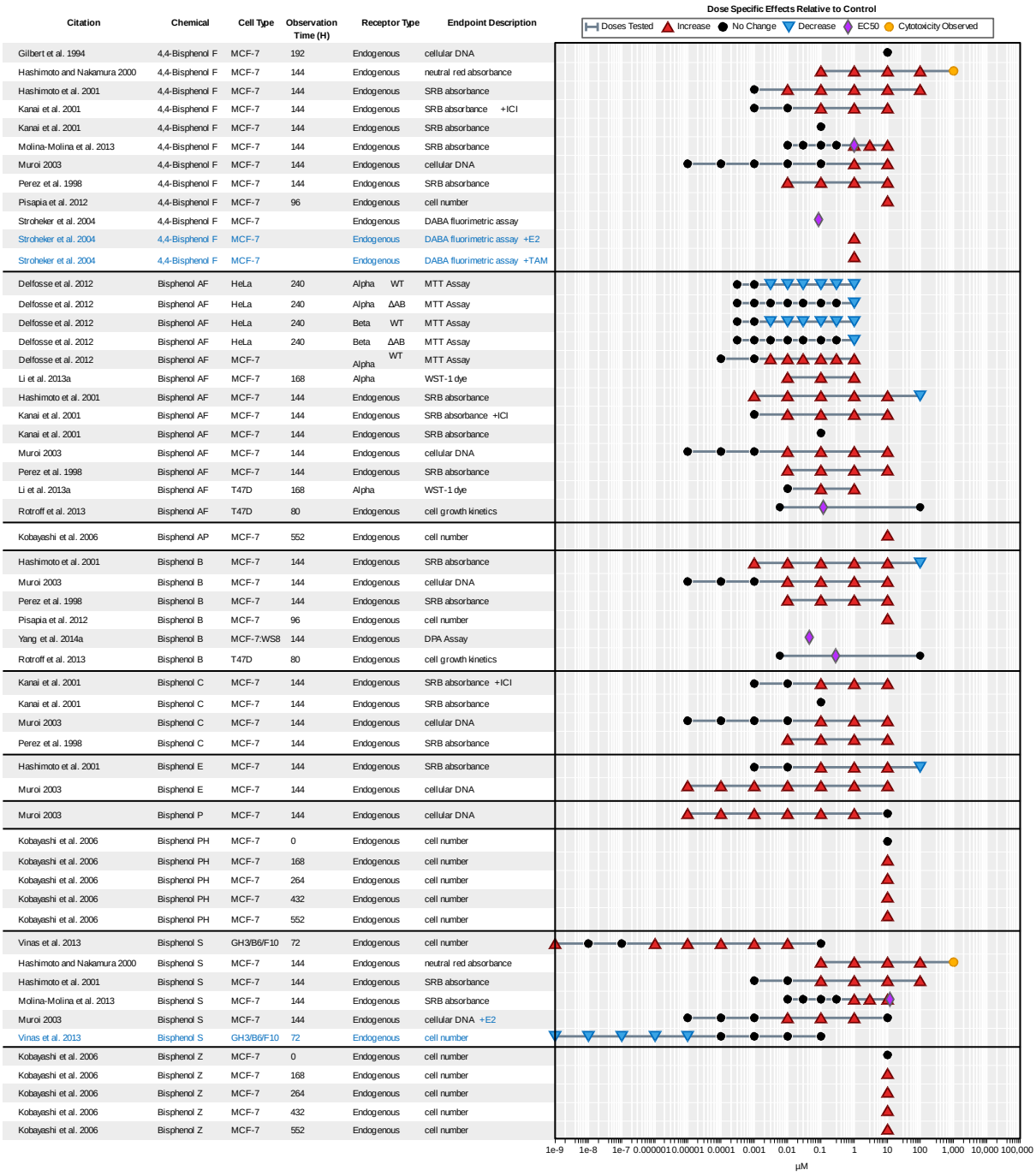
Supplemental Figure 4. Data Pivot of Uterotrophic Response



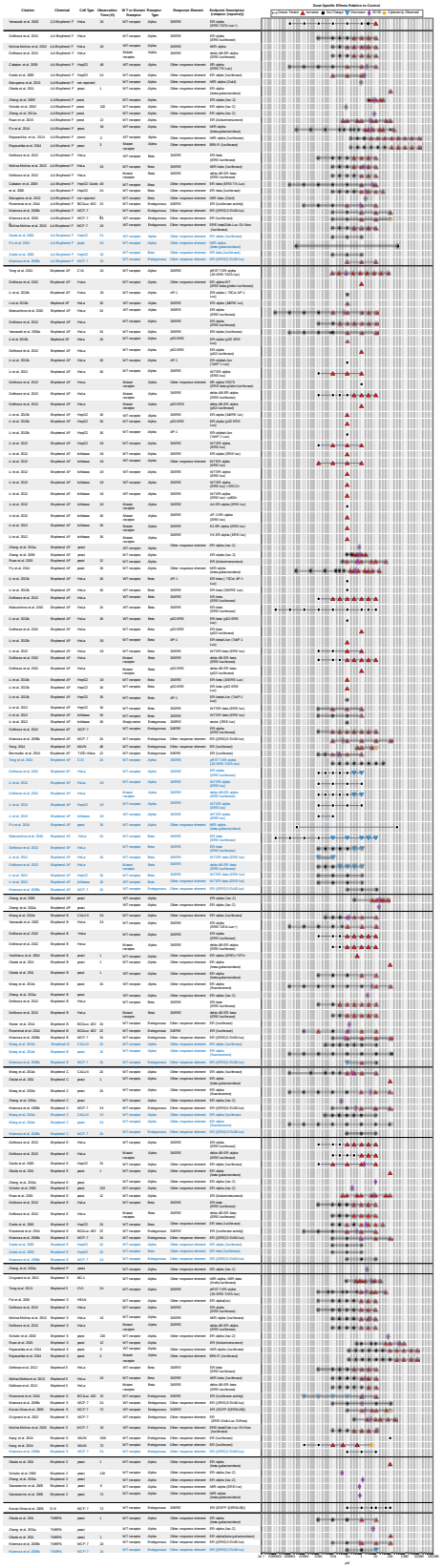
Supplemental Figure 5. Vitellogenin Response



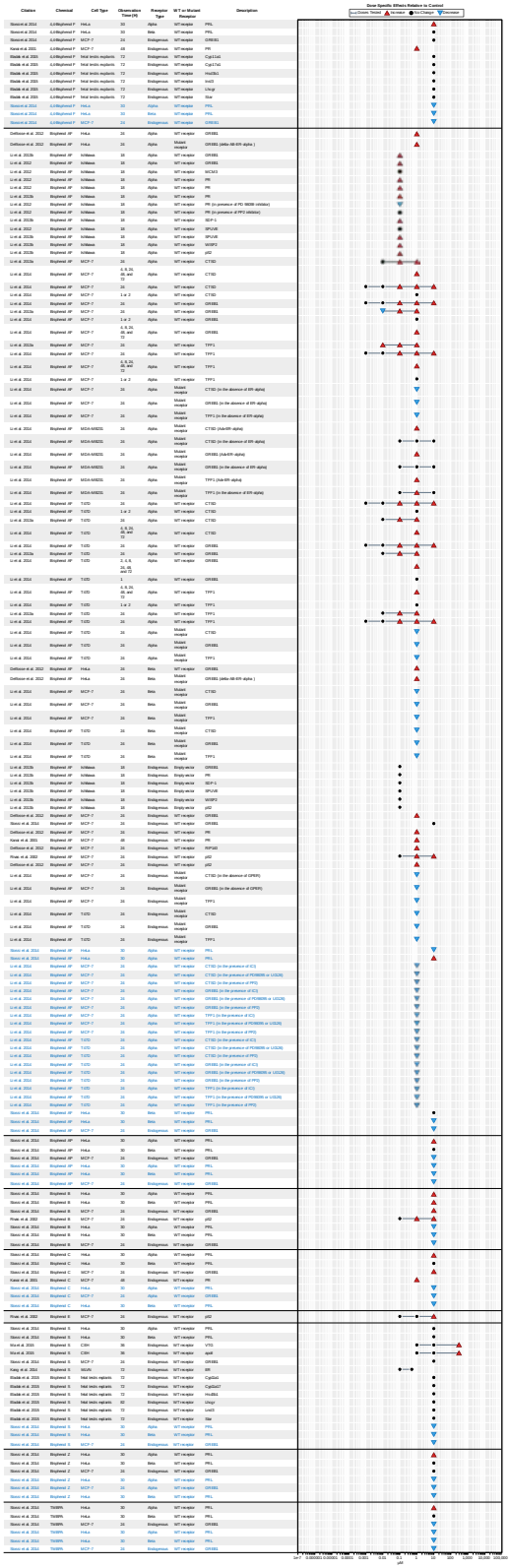
Supplemental Figure 6. Estrogen Receptor Binding Assays



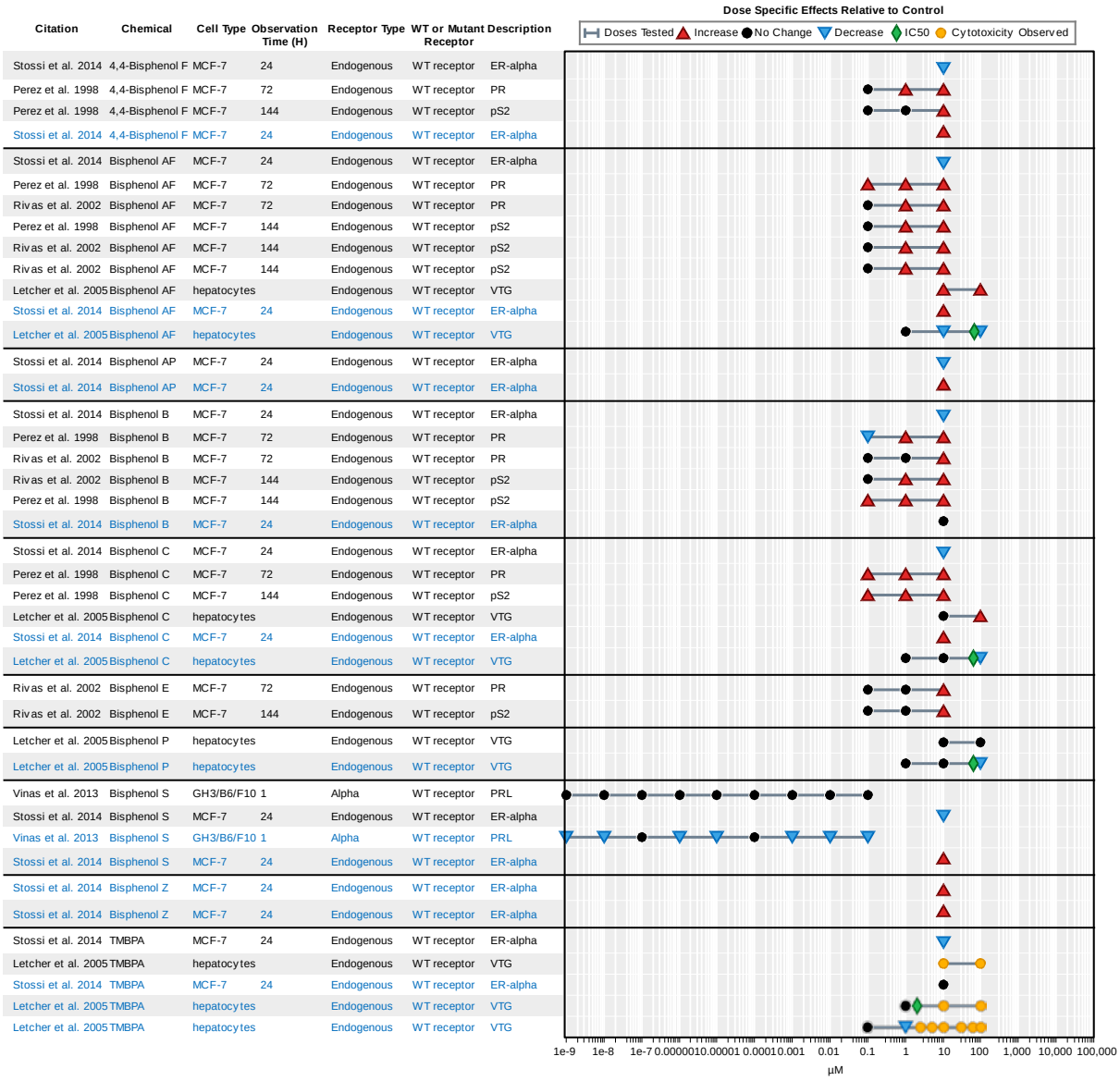
Supplemental Figure 7. [Estrogen Receptor Cell Proliferation Assays]



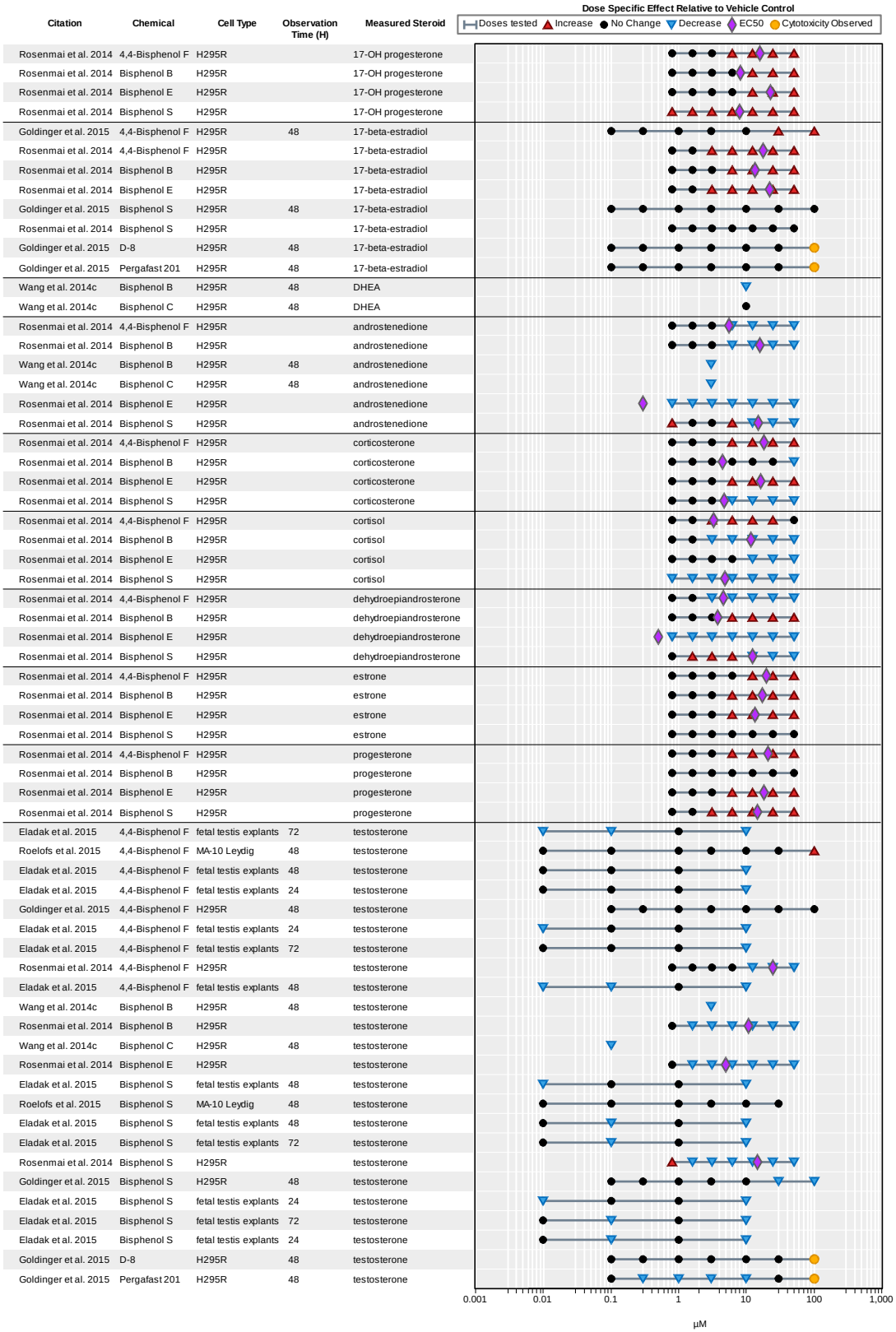
Supplemental Figure 8. [Estrogen Receptor Reporter Gene Assays]



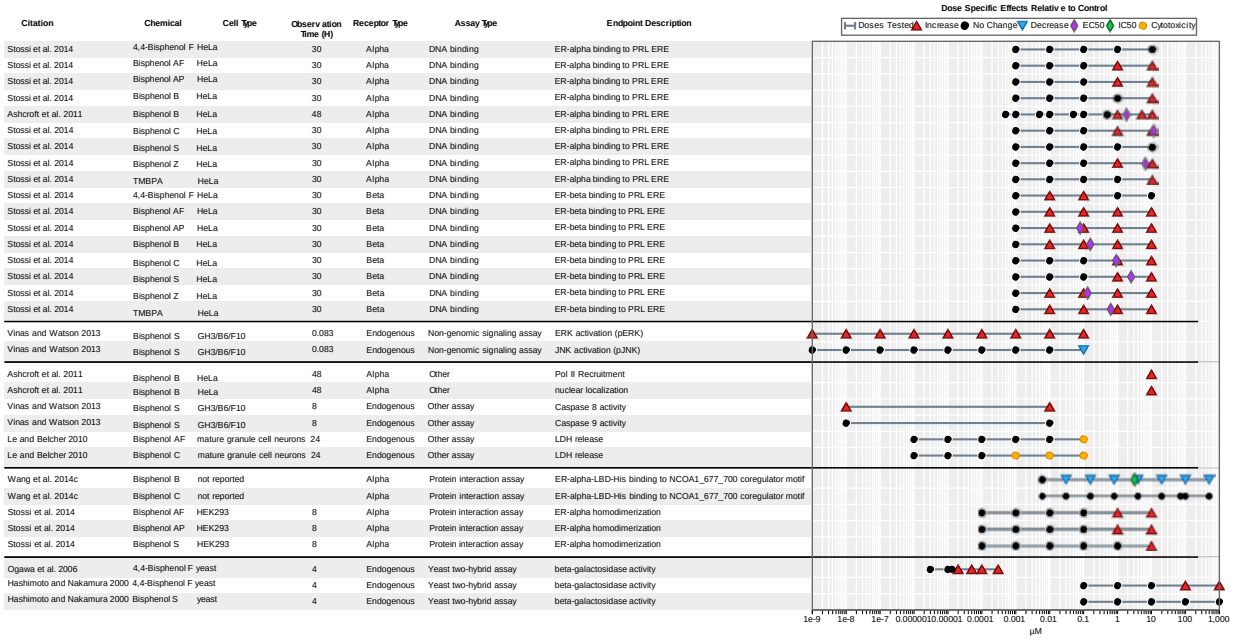
Supplemental Figure 9. [Estrogen Receptor Modulation of Endogenous Gene Expression]



Supplemental Figure 10. Estrogen Receptor Modulation of Protein Expression

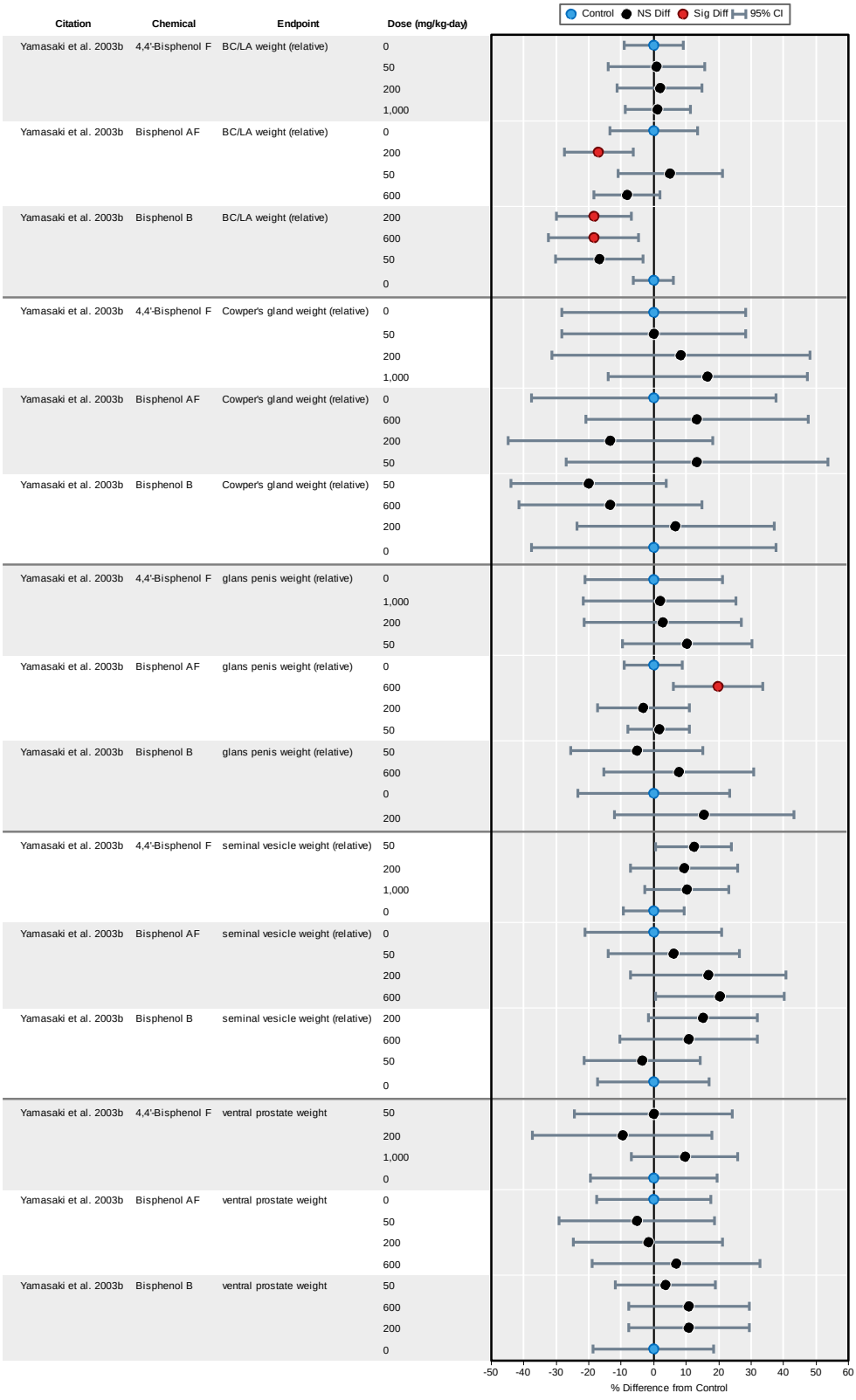


Supplemental Figure 11. Steroidogenesis Assays



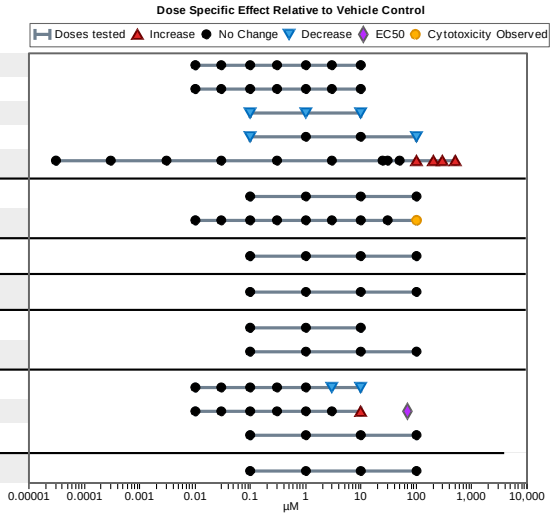
Citation	Chemical	Cell Type	Observation Time (H)	Receptor Type	Assay Type	Endpoint Description
Stossi et al. 2014	4,4-Bisphenol F	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	Bisphenol AF	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	Bisphenol AP	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	Bisphenol B	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Ashcroft et al. 2011	Bisphenol B	HeLa	48	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	Bisphenol C	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	Bisphenol S	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	Bisphenol Z	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	TMBPA	HeLa	30	Alpha	DNA binding	ER-alpha binding to PRL ERE
Stossi et al. 2014	4,4-Bisphenol F	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	Bisphenol AF	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	Bisphenol AP	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	Bisphenol B	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	Bisphenol C	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	Bisphenol S	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	Bisphenol Z	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Stossi et al. 2014	TMBPA	HeLa	30	Beta	DNA binding	ER-beta binding to PRL ERE
Vinas and Watson 2013	Bisphenol S	GH3/B6/F10	0.083	Endogenous	Non-genomic signaling assay	ERK activation (pERK)
Vinas and Watson 2013	Bisphenol S	GH3/B6/F10	0.083	Endogenous	Non-genomic signaling assay	JNK activation (pJNK)
Ashcroft et al. 2011	Bisphenol B	HeLa	48	Alpha	Other	Pol II Recruitment
Ashcroft et al. 2011	Bisphenol B	HeLa	48	Alpha	Other	nuclear localization
Vinas and Watson 2013	Bisphenol S	GH3/B6/F10	8	Endogenous	Other assay	Caspase 8 activity
Vinas and Watson 2013	Bisphenol S	GH3/B6/F10	8	Endogenous	Other assay	Caspase 9 activity
Le and Belcher 2010	Bisphenol AF	mature granule cell neurons	24	Endogenous	Other assay	LDH release
Le and Belcher 2010	Bisphenol C	mature granule cell neurons	24	Endogenous	Other assay	LDH release
Wang et al. 2014c	Bisphenol B	not reported		Alpha	Protein interaction assay	ER-alpha-LBD-His binding to NCOA1_677_700 coregulator motif
Wang et al. 2014c	Bisphenol C	not reported		Alpha	Protein interaction assay	ER-alpha-LBD-His binding to NCOA1_677_700 coregulator motif
Stossi et al. 2014	Bisphenol AF	HEK293	8	Alpha	Protein interaction assay	ER-alpha homodimerization
Stossi et al. 2014	Bisphenol AP	HEK293	8	Alpha	Protein interaction assay	ER-alpha homodimerization
Stossi et al. 2014	Bisphenol S	HEK293	8	Alpha	Protein interaction assay	ER-alpha homodimerization
Ogawa et al. 2006	4,4-Bisphenol F	yeast	4	Endogenous	Yeast two-hybrid assay	beta-galactosidase activity
Hashimoto and Nakamura 2000	4,4-Bisphenol F	yeast	4	Endogenous	Yeast two-hybrid assay	beta-galactosidase activity
Hashimoto and Nakamura 2000	Bisphenol S	yeast	4	Endogenous	Yeast two-hybrid assay	beta-galactosidase activity

Supplemental Figure 12. [Estrogen Receptor Other Endpoints]

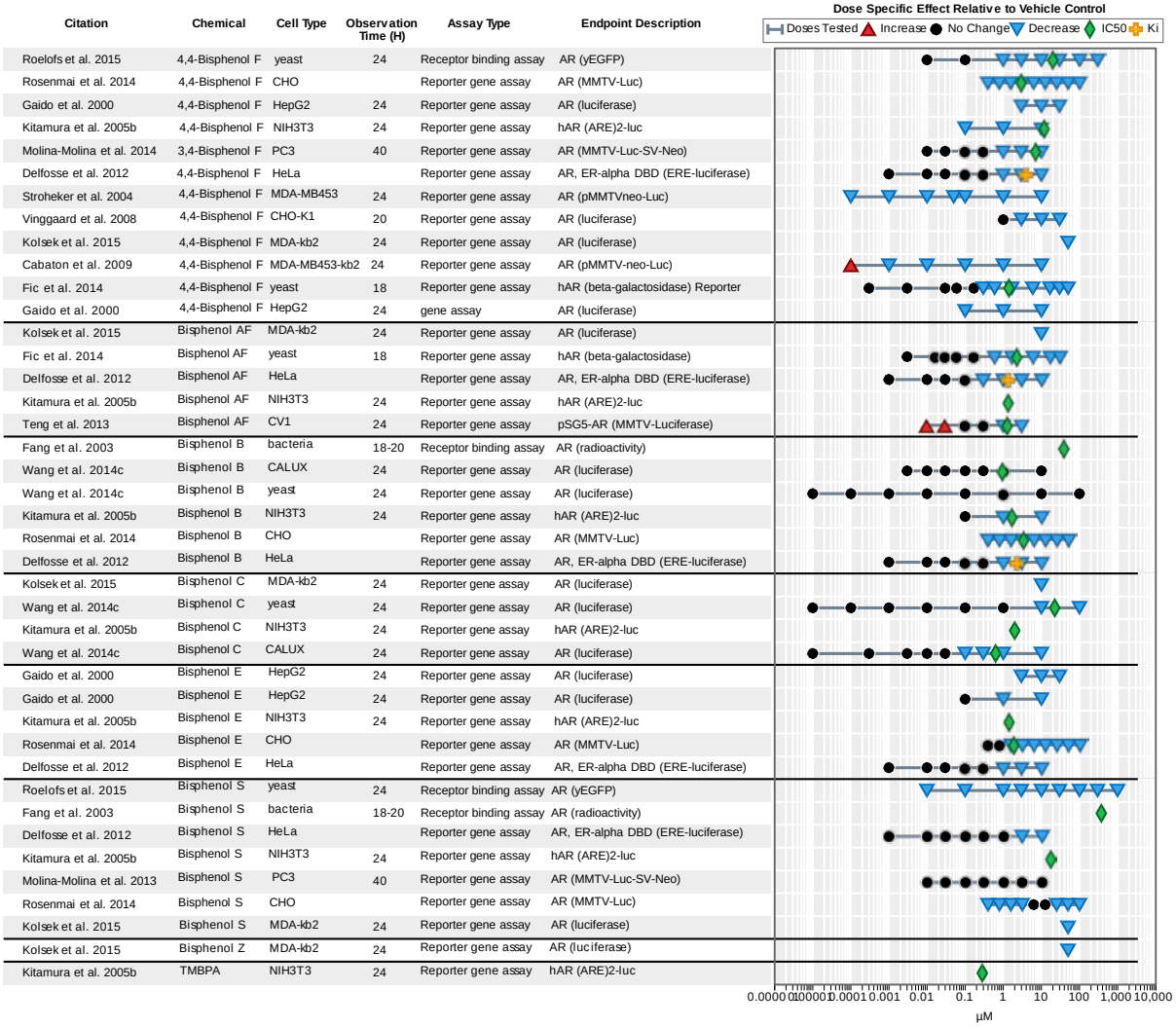


Supplemental Figure 13. [Data Pivot of Hershberger Assay]

Citation	Chemical	Cell Type	Observation Time (H)	Assay Type	Endpoint Description
Molina-Molina et al. 2013	4,4-Bisphenol F	MCF-7	120	Proliferation assay	WT AR (SRB absorbance)
Molina-Molina et al. 2013	4,4-Bisphenol F	PC3	40	Reporter gene assay	AR (MMTV-Luc-SV-Neo)
Gaido et al. 2000	4,4-Bisphenol F	HepG2	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	4,4-Bisphenol F	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Fic et al. 2014	4,4-Bisphenol F	yeast	18	Reporter gene assay	hAR (beta-galactosidase)
Kitamura et al. 2005b	Bisphenol AF	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Teng et al. 2013	Bisphenol AF	CV1	24	Reporter gene assay	pSG5-AR (MMTV-Luciferase)
Kitamura et al. 2005b	Bisphenol B	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Kitamura et al. 2005b	Bisphenol C	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Gaido et al. 2000	Bisphenol E	HepG2	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	Bisphenol E	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Molina-Molina et al. 2013	Bisphenol S	MCF-7	120	Proliferation assay	WT AR (SRB absorbance)
Molina-Molina et al. 2013	Bisphenol S	PC3	40	Reporter gene assay	AR (MMTV-Luc-SV-Neo)
Kitamura et al. 2005b	Bisphenol S	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Kitamura et al. 2005b	TMBPA	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc

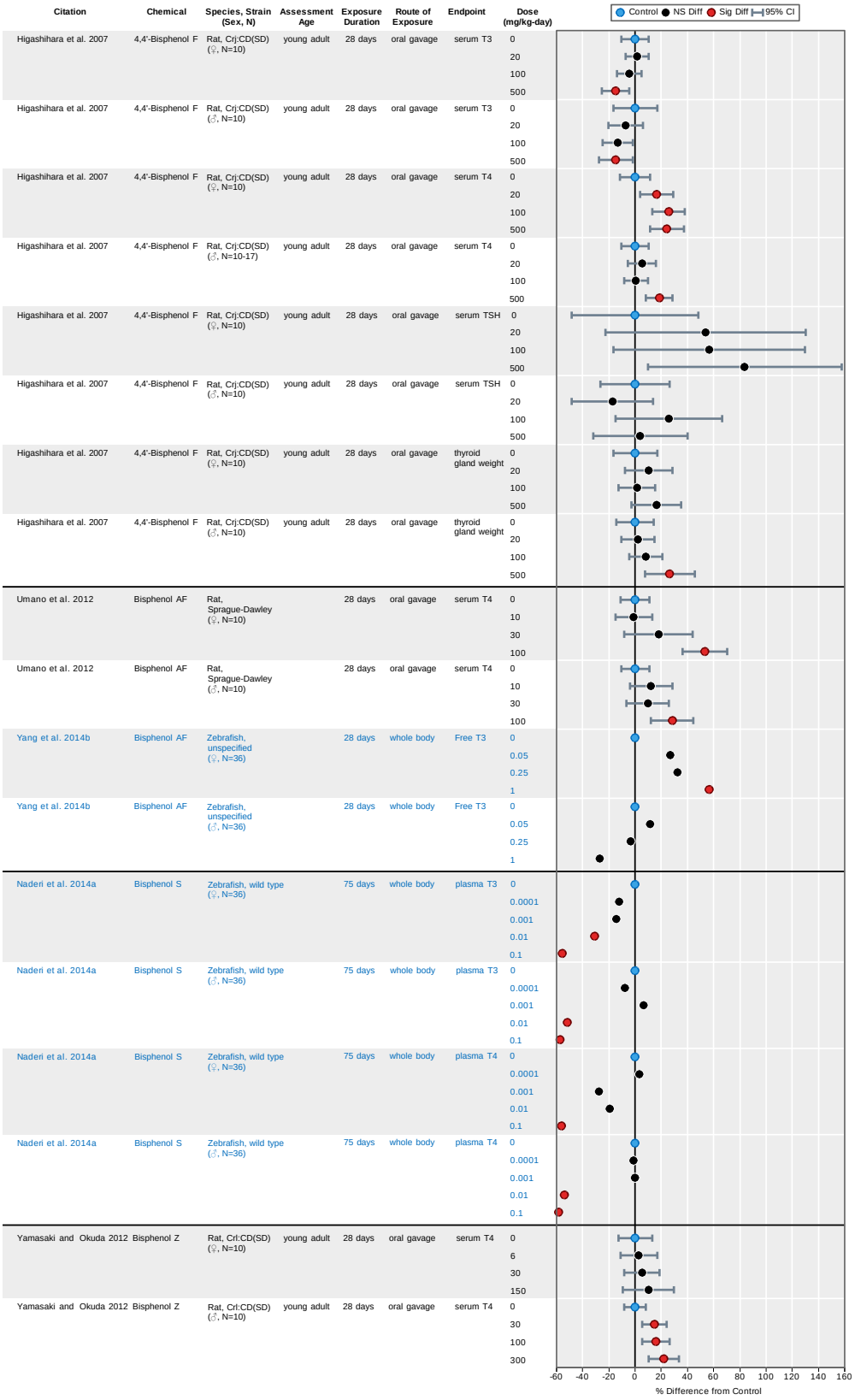


Supplemental Figure 14. Androgen Receptor Agonism Assays



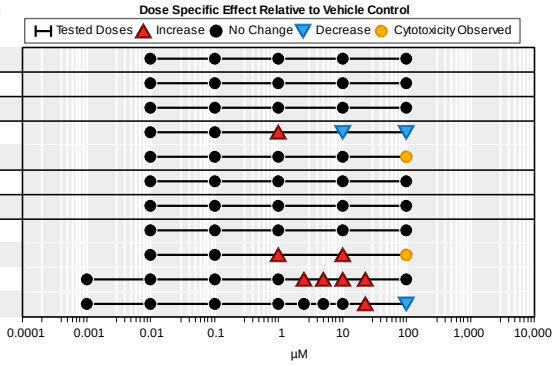
Citation	Chemical	Cell Type	Observation Time (H)	Assay Type	Endpoint Description
Roelofs et al. 2015	4,4-Bisphenol F	yeast	24	Receptor binding assay	AR (yEGFP)
Rosenmai et al. 2014	4,4-Bisphenol F	CHO		Reporter gene assay	AR (MMTV-Luc)
Gaido et al. 2000	4,4-Bisphenol F	HepG2	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	4,4-Bisphenol F	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Molina-Molina et al. 2014	3,4-Bisphenol F	PC3	40	Reporter gene assay	AR (MMTV-Luc-SV-Neo)
Delfosse et al. 2012	4,4-Bisphenol F	HeLa		Reporter gene assay	AR, ER-alpha DBD (ERE-luciferase)
Stroheker et al. 2004	4,4-Bisphenol F	MDA-MB453	24	Reporter gene assay	AR (pMMTVneo-Luc)
Vinggaard et al. 2008	4,4-Bisphenol F	CHO-K1	20	Reporter gene assay	AR (luciferase)
Kolsek et al. 2015	4,4-Bisphenol F	MDA-kb2	24	Reporter gene assay	AR (luciferase)
Cabaton et al. 2009	4,4-Bisphenol F	MDA-MB453-kb2	24	Reporter gene assay	AR (pMMTV-neo-Luc)
Fic et al. 2014	4,4-Bisphenol F	yeast	18	Reporter gene assay	hAR (beta-galactosidase) Reporter
Gaido et al. 2000	4,4-Bisphenol F	HepG2	24	gene assay	AR (luciferase)
Kolsek et al. 2015	Bisphenol AF	MDA-kb2	24	Reporter gene assay	AR (luciferase)
Fic et al. 2014	Bisphenol AF	yeast	18	Reporter gene assay	hAR (beta-galactosidase)
Delfosse et al. 2012	Bisphenol AF	HeLa		Reporter gene assay	AR, ER-alpha DBD (ERE-luciferase)
Kitamura et al. 2005b	Bisphenol AF	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Teng et al. 2013	Bisphenol AF	CV1	24	Reporter gene assay	pSG5-AR (MMTV-Luciferase)
Fang et al. 2003	Bisphenol B	bacteria	18-20	Receptor binding assay	AR (radioactivity)
Wang et al. 2014c	Bisphenol B	CALUX	24	Reporter gene assay	AR (luciferase)
Wang et al. 2014c	Bisphenol B	yeast	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	Bisphenol B	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Rosenmai et al. 2014	Bisphenol B	CHO		Reporter gene assay	AR (MMTV-Luc)
Delfosse et al. 2012	Bisphenol B	HeLa		Reporter gene assay	AR, ER-alpha DBD (ERE-luciferase)
Kolsek et al. 2015	Bisphenol C	MDA-kb2	24	Reporter gene assay	AR (luciferase)
Wang et al. 2014c	Bisphenol C	yeast	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	Bisphenol C	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Wang et al. 2014c	Bisphenol C	CALUX	24	Reporter gene assay	AR (luciferase)
Gaido et al. 2000	Bisphenol E	HepG2	24	Reporter gene assay	AR (luciferase)
Gaido et al. 2000	Bisphenol E	HepG2	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	Bisphenol E	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Rosenmai et al. 2014	Bisphenol E	CHO		Reporter gene assay	AR (MMTV-Luc)
Delfosse et al. 2012	Bisphenol E	HeLa		Reporter gene assay	AR, ER-alpha DBD (ERE-luciferase)
Roelofs et al. 2015	Bisphenol S	yeast	24	Receptor binding assay	AR (yEGFP)
Fang et al. 2003	Bisphenol S	bacteria	18-20	Receptor binding assay	AR (radioactivity)
Delfosse et al. 2012	Bisphenol S	HeLa		Reporter gene assay	AR, ER-alpha DBD (ERE-luciferase)
Kitamura et al. 2005b	Bisphenol S	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc
Molina-Molina et al. 2013	Bisphenol S	PC3	40	Reporter gene assay	AR (MMTV-Luc-SV-Neo)
Rosenmai et al. 2014	Bisphenol S	CHO		Reporter gene assay	AR (MMTV-Luc)
Kolsek et al. 2015	Bisphenol S	MDA-kb2	24	Reporter gene assay	AR (luciferase)
Kolsek et al. 2015	Bisphenol Z	MDA-kb2	24	Reporter gene assay	AR (luciferase)
Kitamura et al. 2005b	TMBPA	NIH3T3	24	Reporter gene assay	hAR (ARE)2-luc

Supplemental Figure 15. Androgen Receptor Antagonism Assays



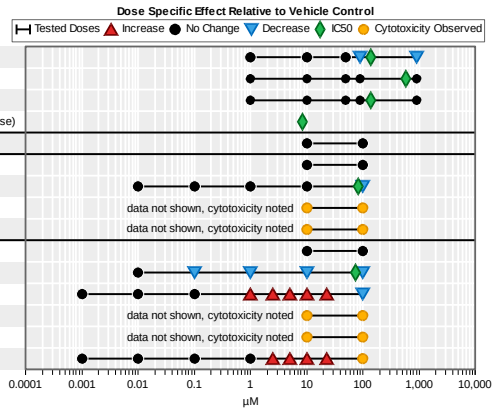
Supplemental Figure 16. Thyroid Hormone Disruption

Citation	Chemical	Cell Type	Observation Time (H)	Assay Type	Endpoint Description
Kitamura et al. 2005b	4,4-Bisphenol F	GH3	48	Protein production assay	GH
Kitamura et al. 2005b	Bisphenol AF	GH3	48	Protein production assay	GH
Kitamura et al. 2005b	Bisphenol B	GH3	48	Protein production assay	GH
Kim et al. 2006	Bisphenol C	GH3	120	Proliferation assay	GH3 cell proliferation
Kitamura et al. 2005b	Bisphenol C	GH3	48	Protein production assay	GH
Kitamura et al. 2005b	Bisphenol E	GH3	48	Protein production assay	GH
Kitamura et al. 2005b	Bisphenol S	GH3	48	Protein production assay	GH
Kim et al. 2006	TMBPA	GH3	120	Proliferation assay	GH3 cell proliferation
Kitamura et al. 2005b	TMBPA	GH3	48	Protein production assay	GH
Shiizaki et al. 2010	TMBPA	yeast	16	Reporter gene assay	TRβ (SRC-1)
Shiizaki et al. 2010	TMBPA	yeast	16	Reporter gene assay	TRα (SRC-1)

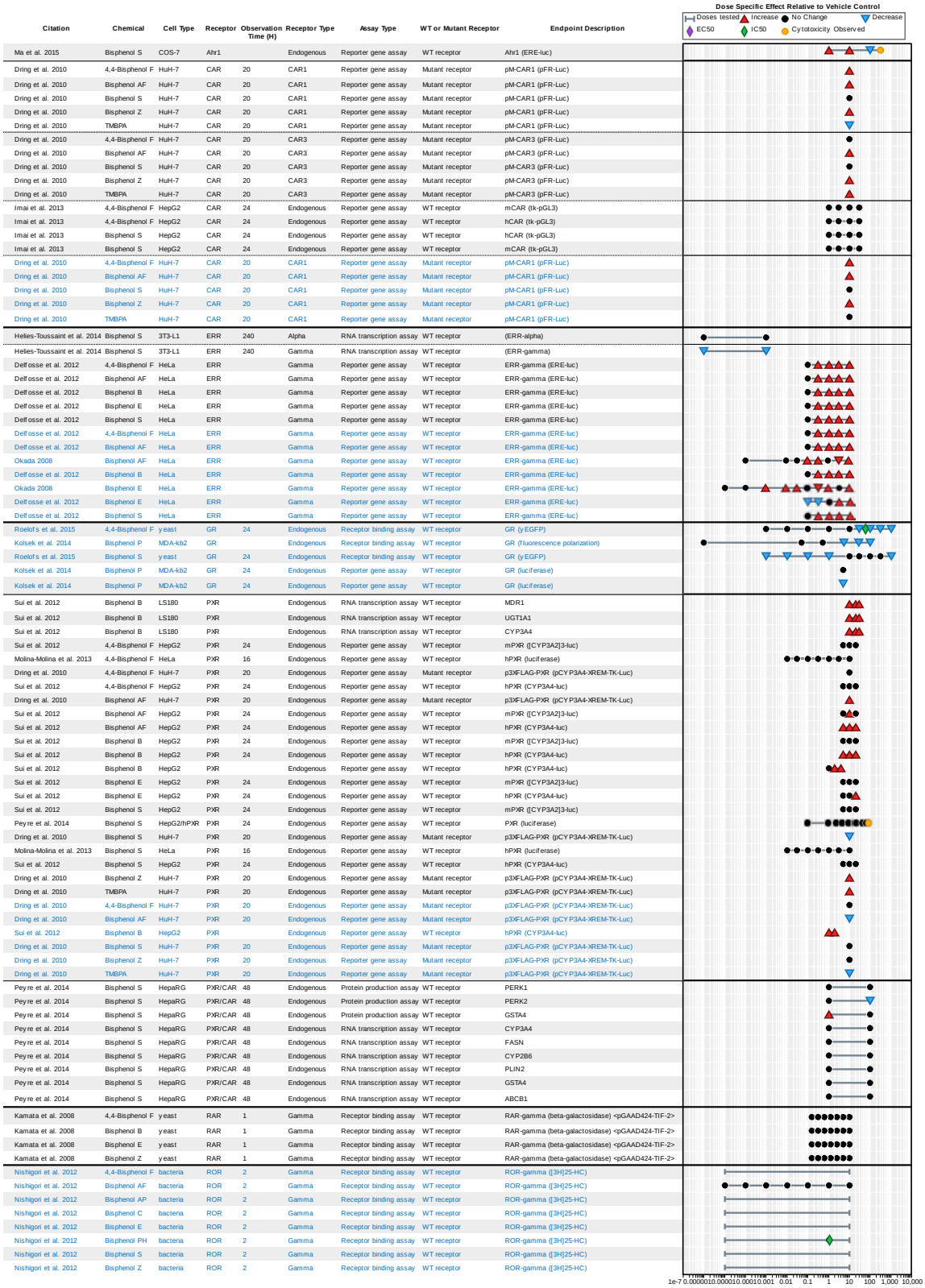


Supplemental Figure 17. Thyroid Receptor Agonism Assays

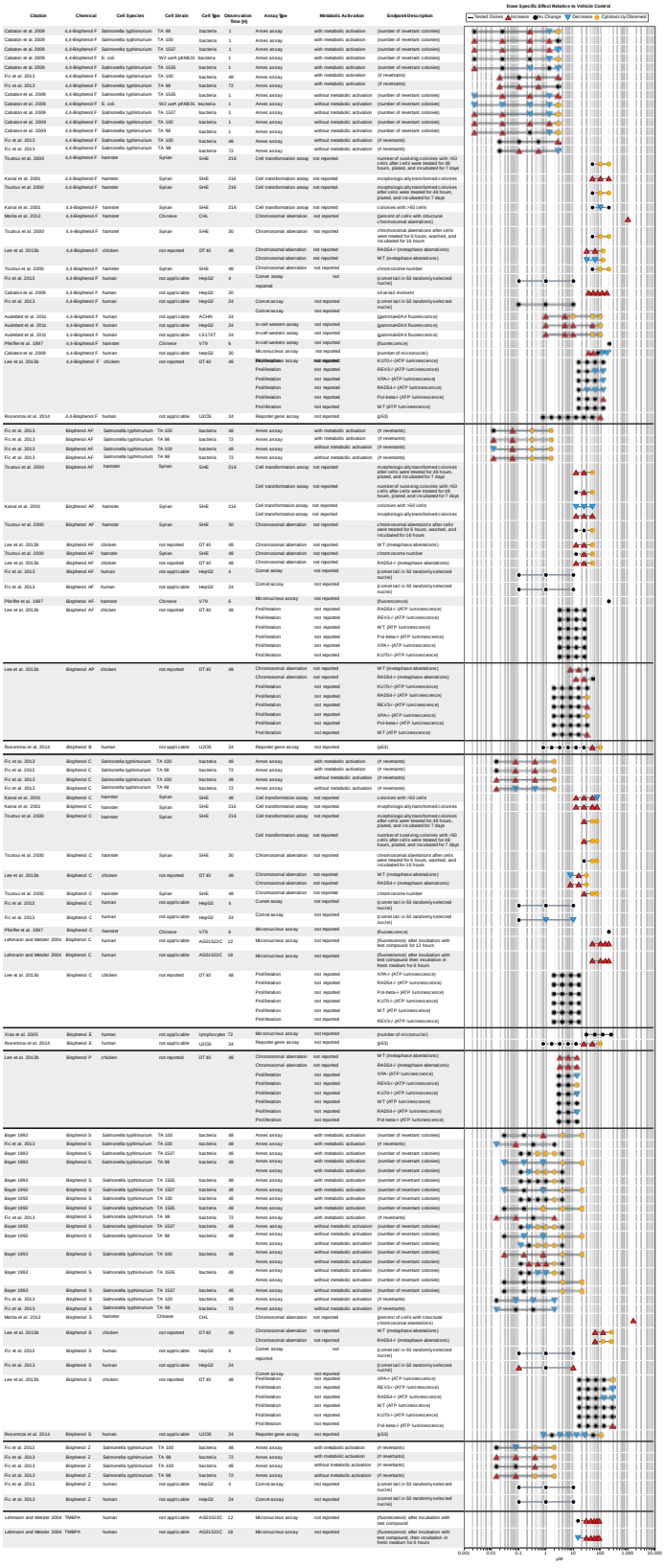
Citation	Chemical	Cell Type	Observation Time (H)	Assay Type	Endpoint Description
Butt et al. 2011	Bisphenol AF	microsomes	1	Enzyme inhibition	deiodinase inhibition (T3 deiodinase)
Butt et al. 2011	Bisphenol AF	microsomes	1	Enzyme inhibition	deiodinase inhibition (rT3 deiodinase)
Butt et al. 2011	Bisphenol AF	microsomes	1	Enzyme inhibition	deiodinase inhibition (3,3'-T2 deiodinase)
Butt and Stapelton 2013	Bisphenol AF	cytosol	0.5	Enzyme inhibition	sulfotransferase inhibition (3,3'-T2 sulfotransferase)
Kitamura et al. 2005b	Bisphenol B	GH3	48	Protein production assay	GH
Kitamura et al. 2005b	Bisphenol C	GH3	48	Protein production assay	GH
Kitamura et al. 2005a	Bisphenol C	MtT/E-2	40	Receptor binding assay	endogenous receptor
Kitamura et al. 2005a	Bisphenol C	CHO-K1	24	Reporter gene assay	TRα
Kitamura et al. 2005a	Bisphenol C	CHO-K1	24	Reporter gene assay	TRβ
Kitamura et al. 2005b	TMBPA	GH3	48	Protein production assay	GH
Kitamura et al. 2005a	TMBPA	MtT/E-2	40	Receptor binding assay	endogenous receptor
Shiizaki et al. 2010	TMBPA	yeast	16	Reporter gene assay	TRβ (SRC-1)
Kitamura et al. 2005a	TMBPA	CHO-K1	24	Reporter gene assay	TRβ
Kitamura et al. 2005a	TMBPA	CHO-K1	24	Reporter gene assay	TRα
Shiizaki et al. 2010	TMBPA	yeast	16	Reporter gene assay	TRα (SRC-1)



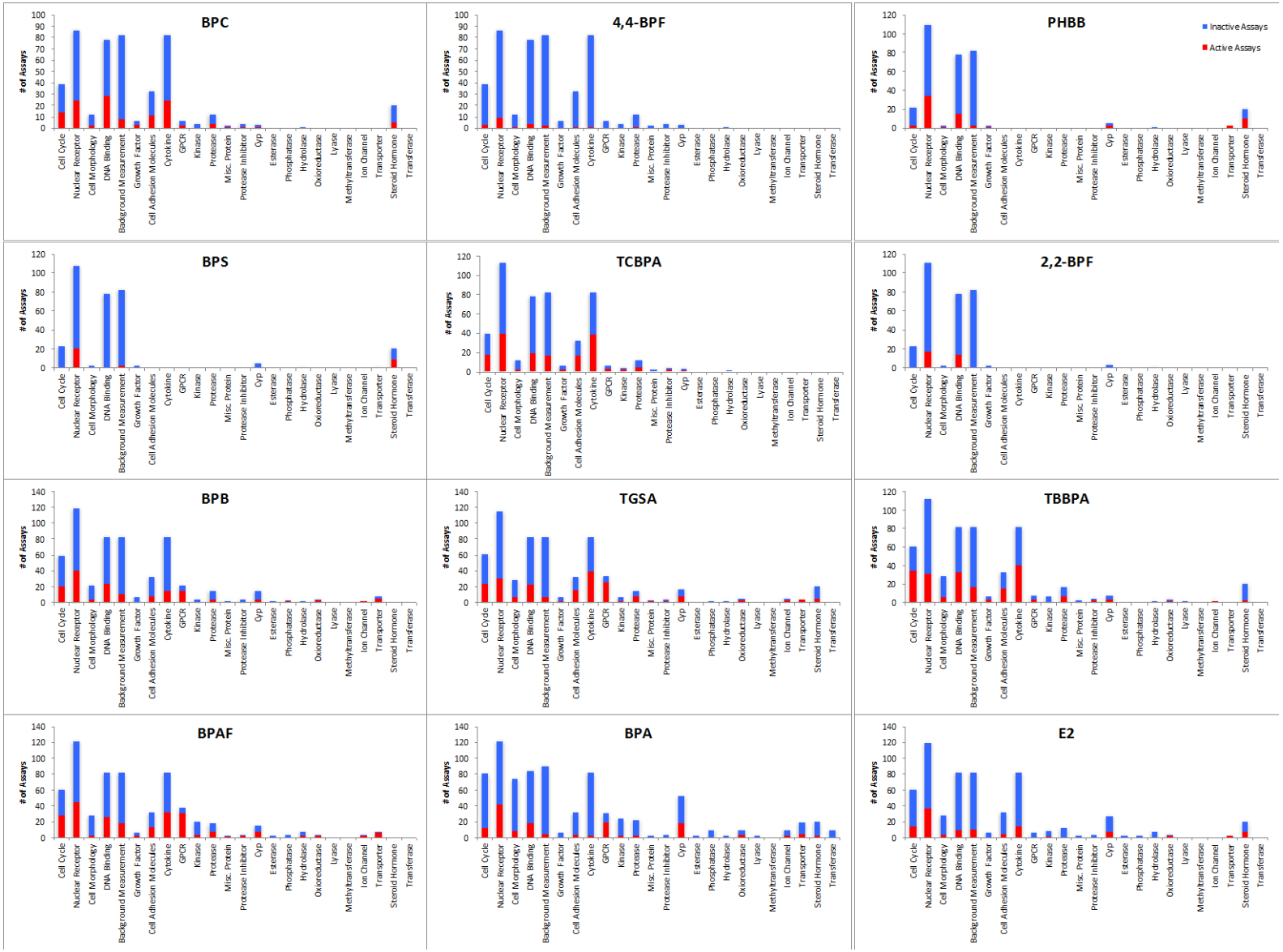
Supplemental Figure 18. Thyroid Receptor Antagonism Assays



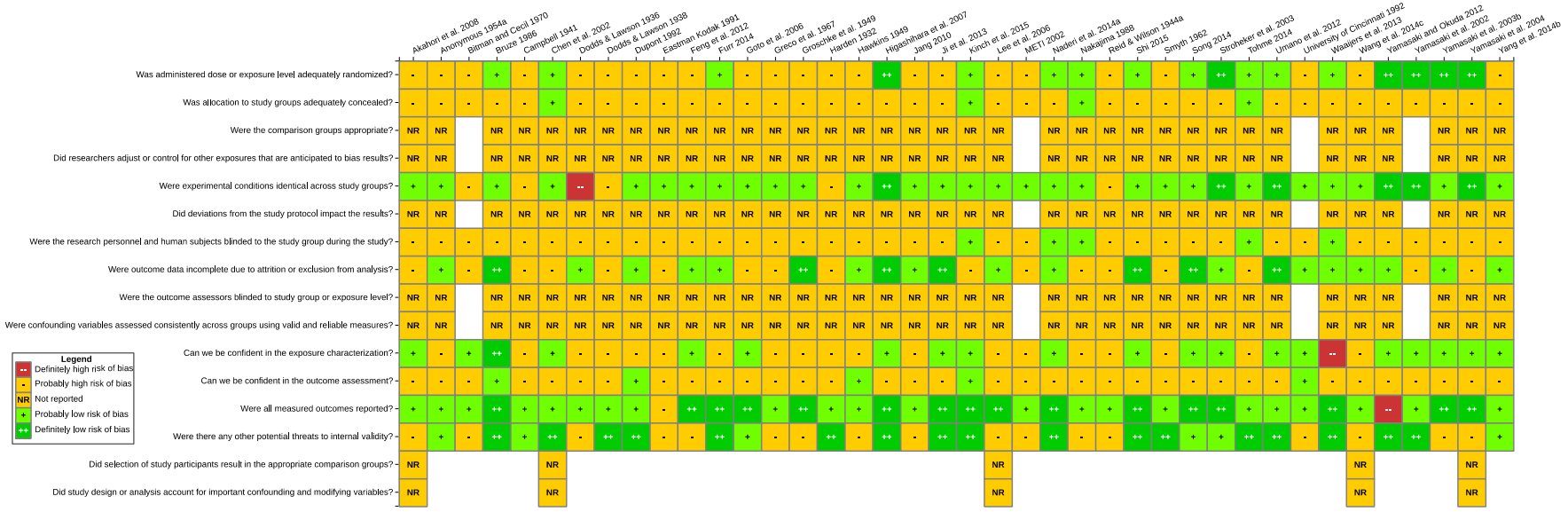
Supplemental Figure 19. Other Nuclear Receptor Activities



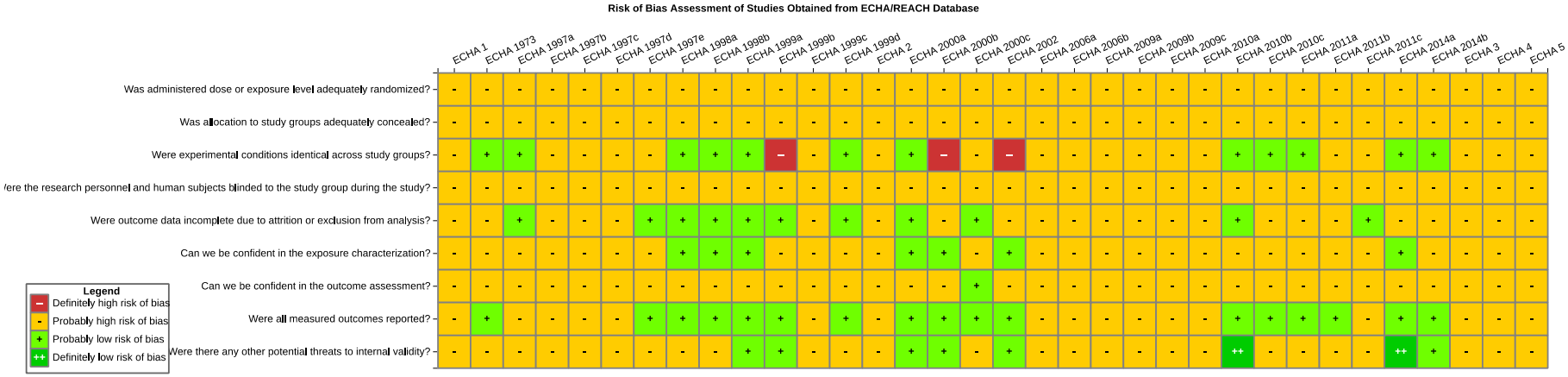
Supplemental Figure 20. *In Vitro* Genotoxicity Assays



Supplemental Figure 21. Assessment of Active and Inactive Assay of BPA Analogues in ToxCast



Supplemental Figure 22. [Risk of Bias of Animal Evidence from Published Literature]



Supplemental Figure 23. Risk of Bias of Animal Evidence from ECHA Data

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